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Identification of Novel Epigenetic Biomarkers for the Early Diagnosis of Colorectal Cancer

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ABSTRACT

Colorectal cancer (CRC) is one of the leading causes of cancer death worldwide. Currently, no effective early diagnostic biomarkers are available for colorectal carcinoma. Therefore, there is a need to discover new potential biomarkers for the early diagnosis of this tumor or pre-cancerous lesions.

Several studies have demonstrated the diagnostic potential of microRNAs, a class of small non-coding RNA of 20-22 nucleotides in length, whose dysregulation has been already associated with the development of different pathologies.

On these bases, the aim of the present study was to computationally identify a set of miRNAs associated with the development and progression of colorectal cancer in order to validate their diagnostic and prognostic potential in a pilot cohort of CRC patients and healthy controls. In particular, first a bioinformatics analysis was performed by analyzing the computational data contained in The Cancer Genome Atlas (TCGA) and GEO DataSets databases in order to identify a list of miRNAs associated with the development of colorectal cancer. Further bioinformatics prediction tools were used to establish the functional role of these miRNAs in colorectal cancer. Subsequently, the *in silico* data were validated in both FFPE and liquid biopsy samples obtained from colorectal cancer patients and healthy controls by using the high-sensitive droplet digital PCR (ddPCR) amplification systems. After ddPCR analyses, the diagnostic potential of four selected miRNAs, hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-503-5p and hsa-miR-375 was assessed through statistical analyses and further bioinformatics evaluations.

The bioinformatics analyses allowed to identify a set of 19 miRNAs significantly dysregulated in colorectal cancer patients and thus potentially useful as diagnostic biomarkers for this pathology. Further computational approaches allowed us to select the four miRNAs hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-503-5p and hsa-miR-375 in order to validate their diagnostic and prognostic potential on FFPE and liquid biopsy samples.

The ddPCR analyses revealed that the expression levels of hsa-miR-21-5p and hsa-miR-503-5p were significantly increased in colorectal cancer FFPE samples

compared to the normal adjacent mucosa while the expression levels of hsa-miR-375 and hsa-miR-497-5p were significantly down-regulated in tumor tissues.

Weaker results were obtained for the liquid biopsy samples where only hsa-miR-21-5p expression levels increased significantly in tumor samples and only hsa-miR-497-5p showed a significant decrement of the circulating levels in tumor patients.

After validating the diagnostic potential of the miRNAs here identified, the results of further computational analyses allowed us to establish also the prognostic value of the selected miRNAs. In particular, the circulating levels of hsa-miR-375 can be used as a reliable prognostic biomarker to establish the overall survival of patients. Similarly, the tissue levels of hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375 can predict the overall survival rate of patients at the diagnosis.

Overall, the results obtained confirmed the bioinformatics results previously observed. Therefore, this study represents the starting point for the adoption of ddPCR for the effective and sensitive analysis of miRNA expression levels both in tissue and liquid biopsy samples. Thus, this strategy could be applied for the non-invasive early diagnosis of colorectal cancer for individuals at risk for this tumor.

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1. INTRODUCTION

Colorectal cancer (CRC) represents the most frequent neoplasm of the digestive tract. According to the epidemiological data collected worldwide, colorectal cancer accounts for the second most diagnosed tumor in women and the third tumor in males. This tumor is frequently diagnosed in elderly people however there are familial syndromes with early onset [Siegel RL et al, 2020]. Despite the numerous therapeutic strategies available for this tumor, colorectal cancer is still responsible for a significant number of cancer death annually ranging the fourth cause of mortality worldwide [Rawla P et al, 2019].

Numerous factors, mainly related to dietary and lifestyle habits are associated with the onset of colorectal cancer. Among these risk factors, a prominent role is played by obesity, cigarette smoking, alcohol consumption and bad dietary habits [Johnson CM et al, 2013]. In addition to these risk factors, there are also several physiological and pathological predisposing conditions, such as age over 50 years, family history of CRC, inflammatory bowel disease (IBD) and inherited syndromes (Lynch syndrome, Familial adenomatous polyposis, etc.) [Hnatyszyn A et al, 2019].

Besides these environmental risk factors, the development of colorectal cancer is associated with molecular alterations affecting key genes involved in the alteration of cellular homeostasis. Among these, mutations affecting KRAS, APC, TP53 and PIK3CA genes are the most frequently described in colorectal cancer patients [El Bali M et al, 2021]. Other alterations affect the Mismatch Repair Mechanisms thus leading to an accumulation of somatic mutations [Hou JT et al, 2018].

During the last years, several studies have tried to identify potential biomarkers for the early diagnosis and for the management of this tumor, however, no convincing results have been obtained.

On these bases, the discovery of new markers and the implementation of new diagnostic strategies for the early identification of colorectal cancer may improve the strategies available for the management of this tumor.

In the last decade, several studies focused their attention on the potential pathogenetic and diagnostic role of microRNAs (miRNAs), small non-coding RNA sequences able to inhibit gene expression by binding to mRNAs. As widely described below, miRNAs can be easily detected in different biological samples, including serum, saliva and stool, and used as biomarkers with high specificity and sensitivity for the identification of pre-cancerous lesions [Falzone L et al, 2020; Crimi S et al, 2020]. However, conflicting data were generated on this matter. Such contradictory results may be caused by the difficulty in analyzing the huge amount of information available derived from the genome sequencing analysis.

In this context, the identification of novel biomarkers associated with the development of colorectal cancer is a hot topic of cancer research. Therefore, computational and experimental studies aimed at identifying novel biomarkers for colorectal cancer are mandatory to improve the management of this tumor.

1.1 Epidemiology

Colorectal cancer was the most common cause of cancer death in the United States in the late 1940s and early 1950s [SEER Database, 2013].

Today, colorectal cancer (CRC) represents the third most common malignant cancer in both sex in European countries [Siegel RL et al, 2013; Lech G et al, 2016] in part because of historical changes in risk factors (e.g., decreased smoking and red meat consumption), the introduction and dissemination of early detection tests, and improvements in treatment. This malignancy affects more than one million people on an annual basis and it is responsible for more than half a million deaths [Siegel RL et al, 2013; Lech G et al, 2016].

Based on recently available data, the overall five-year survival rate from the diagnosis can achieve 65%, but this percentage can significantly vary depending on stage of malignancy [Lech G et al, 2016]

The larger part of the cases and the most part of the deaths occur in the elderly population (65 years old or more) [SEER Database, 2013].

The lifetime probability of a colorectal cancer diagnosis is 5% in men and 4,7% in women. Also, incidence and mortality rates are 30 to 40% higher in men than

in women overall. Those rates however variate with age. The reasons for this higher incidence in men are not known, but a connection between hormones, lifestyle and eating habits has been hypothesized [SEER Database, 2013; Murphy G et al, 2011; Meissner HI et al, 2006]. A connection between low socioeconomic status and a higher risk of incidence of colorectal cancer and death has been observed [SEER Database, 2013; Albano JD et al, 2007; Doubeni CA et al, 2012].

Clinical and biological characteristics vary in colorectal cancers depending on the site of incidence within the colon and the rectum. This observation suggests distinct etiologies and carcinogenic mechanisms [SEER Database, 2013; Iacopetta B, 2002; Matanoski G et al, 2006; Nawa T et al, 2008]

The proximal colon (caecum and ascending colon) is the most common location of colorectal carcinoma genesis. Cases originating in the proximal colon account for a 42% of the total. The second most affected site is the rectum, with a number of cases accounting for the 28% of the total [SEER Database, 2013]. Also, the percentage of proximal tumors in women is higher than in men (46% vs 38%).

There are also marked differences considering age at diagnosis, with an important increase in proximal tumors and decrease in rectal tumors with advancing age [SEER Database, 2013].

For example, 56% of colorectal cancers in women aged 80 years and older are in the proximal colon, compared with 26% in those aged younger than 50 years. Consequently, the median age at diagnosis for rectal cancer is younger (63 years in men and 65 years in women) than that for colon cancer (69 years and 73 years, respectively) [SEER Database, 2013].

If a colorectal cancer is diagnosed when the disease is at a local stage the 5-year survival rate is high, reaching a percentage of more than 90% of the total diagnosis. However, this survival rate declines to 70,4% and 12,5% when the diagnosis is done in cases with loco-regional diffusion and distant metastasis, respectively Globally, the 5-year survival rate ranges between 28% and 42% in developing countries, while it is superior to 60% when considering data from U.S., Japan and Switzerland [Coleman MP et al, 2008].

In Italy, 5-years survival is about 60,8% for colon and 58,3% for rectum, resulting above the European average (50,7% and 55,8%, respectively).
Over 427.000 patients, in Italy, are diagnosed with colorectal cancer, of which 53% are males (representing 14% of total oncologic patients). 17% of these patients are already over 15 years since diagnosis, while the remaining population sample is equally distributed in other categories: within two years since diagnosis; between 2 and 5 years and between 5 and 10 years. These data are the result of a slight decline in mortality, due to multiple factors: wide-spreading of efficient early screening and its accessibility; increased possibility to obtain effective treatments.
Progressive aging also increases the possibility of comorbidity [Bach PB et al, 2002]. For this reason, the majority of affected people is above 75 years old (2914 cases every 100000 individuals); a ratio that reduces by half in the immediately below age group, between 60-74 years old, and is eight-to-ten time less in age range between 45-59 years of life.

1.2 Macroscopic and Microscopic Features

1.2.1 Anatomy

The large intestine extends from the distal end of the ileum to the anus. It represents the terminal part of the digestive tract. It absorbs fluids and salts from the gut contents, thus forming feces, and consists of the cecum, appendix, colon, rectum, and anal canal.
Colon and rectum together form a tube of variable diameter and they are approximatively 150 cm long.
The terminal ileum encounters the cecum (the first segment of the colon) through a thick invagination called ileo-cecal valvula. Cecum is a segment of the proximal colon characterized by a medium diameter of 7,5 cm and a medium length of 10 cm.
The appendix is a narrow, hollow, blind-ended tube connected to the cecum. It has large aggregations of lymphoid tissue in its walls and is suspended from the terminal ileum by the mesoappendix, which contains the appendicular vessels.

Its point of attachment to the cecum is consistent with the highly visible free taeniae leading directly to the base of the appendix.

Ascending colon, which is approximately 15 cm long, runs towards the liver on the right side of the peritoneal cavity. Its posterior face is fixed to the retroperitoneum, while anterior and lateral surfaces are intraperitoneal structures.

The Toldt fascia is the structure formed by the fusion of the mesentery with the posterior peritoneum and it represents a landmark for the surgeon to perform the mobilization of the colon during resections.

The transverse colon is approximately 45 cm long. It is "suspended" between two fixed positions, which are the hepatic (or right colic flexure) and the splenic flexure (or left colic flexure). The nefrocolic ligament is the structure that sustains the hepatic flexure and it covers the right kidney, the duodenum and the hepatic hilum.

The frenocolic ligament runs towards the spleen and is responsible for the fixation of the splenic flexure in the left upper quadrant.

The descending colon is situated towards the left kidney, and it runs down from the splenic flexure. It is approximately 25 cm long. At the level of the upper pelvis it can be observed a transition zone from the descending colon to the sigmoid colon.

The sigmoid colon is thicker and not fixed as the descending portion. The sigmoid colon length is widely variable (from 15 to 50 cm). Below the sigmoid colon, it can be observed the rectum (which is the portion dedicated to the continence of faces).

The rectum is 12-15 cm long. Its posterior face is almost completely extraperitoneal, due to the fact that it is adherent to the presacral soft tissues external to the peritoneal cavity.

The rectum has three invaginations called Houston valves. After a surgical mobilization of the segment, these three invaginations can add approximately 5 cm more to the surgeon, making easier a possible deep anastomosis. The posterior surface of the rectum is covered by a thick mesorectum. A thin layer

of tissue covers the mesorectum and it is distinct from the pre-sacral fascia on which it is adherent.

During a surgical operation for a rectal tumor, it is important to dissect the entire mesorectum (Total Mesorectal Excision – TME) and it represents a well-described and fundamental surgical maneuver.

The vascularization of colon and rectum is supplied by arteries coming from the descending aorta (mainly superior mesenteric artery (SMA) – responsible for the supply of the right portion of the colon – and inferior mesenteric artery (IMA) – responsible for the vascularization of the left portion).

SMA gives origin to the middle colic artery, the right colic artery and the ileo-cecal artery.

IMA artery gives origin to the left colic artery, to the sigmoid arteries and to the superior rectal artery.

The Riolano marginal artery is a fundamental connection between the system of vascularization of the SMA and of the IMA. It follows the entire length of the colon on the inner side and it is of fundamental importance in case of anastomosis.

The lower part of the rectum is supplied by the middle and inferior rectal arteries, originating from the interior iliac artery.

Venous drainage follows the arterial distribution. This is the cause of the different metastatic diffusion of tumors originating in the upper part of the rectum and of tumors originating in the lower part.

Colorectal cancer (CRC) can affect every part of the colon and rectum.

The behavior of the malignancy is however different, based on the segment affected.

Tumors affecting the right part of the colon cause anemia, pain in the right quadrant, anorexia, while tumors affecting the left part of the colon and the rectum cause modifications of the hive (both stypsis or diarrhea), enterorrhagia, abdominal pain.

When the tumor is located in the left segments of colon, complications such as occlusion or perforation are more common.

1.2.2 Microscopic characteristics

About 85% of CRCs are adenocarcinomas, characterized by hyper-proliferation of cylindric cells that form glandular structures. From a microscopic point of view, it is possible to distinguish between different kinds of adenocarcinomas [Hedinger C et al., 1989]:

- Mucinous adenocarcinoma: characterized by abundant presence of mucinous cells, that constitutes over 50% of total tumor cells. Mucin production promotes invasion and metastasis formation, because of its capability to dissolve muscular fibers of underneath muscular layer.
- Ring shaped-cells carcinoma: these tumor cells present a vacuole in the cytoplasm in which a high amount of mucin is stored, therefore the nucleus is localized in the cell periphery;
- Undifferentiated carcinoma: does not show any sign of typical epithelial differentiation;
- Small-cell carcinoma
- Squamous carcinoma: neoplastic cells show a squamous morphology;
- Adenosquamous carcinoma: neoplastic cells are characterized by mixed morphology between squamous and glandular.

1.2.3 Neoplastic cells' differentiation grade

Grading indicates how many neoplastic cells morphologically diverge from normal cells. It can be evaluated in different ways, analyzing certain parameters, as: nucleus polarity, integrity and definition of glandular structures, growth pattern, inflammatory infiltration and desmoplastic reaction of near tissue. According to these parameters, it is possible to distinguish 4 different grades of cell differentiation:

- Grade 1: Cylindric, well-differentiated cells, similar to normal cells;
- Grade 2: Moderately differentiated neoplasia;
- Grade 3: Poorly differentiated neoplasia;
- Grade 4: Anaplastic neoplasia, the glandular structure is almost totally lost.

1.3 Risk Factors and Protective Factors

As already mentioned, some dietary and lifestyle habits are directly linked with a higher rate of colorectal cancer. In general, colorectal cancer risk factors are classified into modifiable risk factors and unmodifiable risk factors. These latter are mainly associated with inherited genetic alterations responsible for an increased risk of colorectal cancer. Besides these risk factors, some foods or habits are considered protective factors for this tumor

1.3.1 Modifiable risk factors

The main risk factors associated with the development of colorectal are related to several environmental agents:

- Dietary habits: reduced intake of non-absorbable fibers and excessive consumption of refined carbohydrates, fat foods and red meat are all correlated with CRC development.

A poor intake of fibers seems to be correlated with the onset of CRC due to microbial environment composition alteration. The reduction of the fecal mass, caused by the poor assumption of non-absorbable fibers, determines a longest contact-time between toxic subproducts derived by microbial metabolism and colon mucous membrane.

Also the lack of certain vitamins, like A, C and E, determines an increase in oxidative power of these microbial subproducts, as the vitamins are powerful antioxidants [Kumar V et al, 2012].

Fat foods consumption contributes to increase the risk by increasing the hepatic synthesis of cholesterol and bile acids, which are converted into carcinogenic substances by intestinal microbial flora.

In 2015, the International Agency for Research on Cancer of the World Health Organization, classified processed meat as class 1 carcinogen, and red meat in class 2A [Bouvard V et al, 2015]. In fact, a high consumption of red processed meat is correlated to an increased risk of developing distal-colon cancer. The risk increases by 17% per 100g of red meat per day, by 18% per 50g of processed meat (smoked, salted and seasoned) per day [Chao A et al, 2005; Chan DS et al, 2011].

- Cigarette smoke: It may be correlated mostly to the rectal localization of cancer. A meta-analysis based on 106 observational studies determined that smokers have a risk about 2.18, compared with the 1.25 in non-smokers, confirming the correlation between cigarette smoke and colorectal cancer.
- Alcohol: an excessive consumption of alcohol raises the risk of CRC onset. A meta-analysis based on 27 cohort studies and 34 case-control studies determined that medium drinkers (2-3 drinks per day) have a risk equal to 1.21, while in alcohol abusers, this risk grows up to 1.52.

1.3.2 Unmodifiable risk factors: Genetic and epigenetic alterations in colorectal cancer

Genetic susceptibility to CRC, as also hereditary diseases and Inflammatory Bowel Disease (IBD), represent the main risk factors that cannot be modified.
- Genetic predisposition is involved both in familiar (about 30%), non-familiar (about 65%) and in hereditary (less than 5%) cases of CRC. Hereditary CRC can originate from pre-neoplastic damages (e.g. polyp tumor) or directly from mucous membrane. For this reason, it is possible to distinguish between nonpolyposis syndrome and polyposis syndrome. One example of nonpolyposis syndrome is represented by Lynch Syndrome (or hereditary nonpolyposis colorectal cancer), which is caused by mutations that occur in germline, inside genes involved in mismatch repair (MMR) mechanism, like MLH1, PMS1, PMS2, MSH2 and MSH6. The mutations can be inherited with an autosomal dominant model, with a 90% penetrance [Nagy R et al, 2004].
MMR system has the important role of correcting mismatches in base pairs that normally occur in every DNA replication cycle. MMR genes mutation mainly consists of base substitution, small indels (insertions-deletions) that may fall in repeated nucleotides sequences named microsatellite DNA. These mutations in repeated nucleotide sequences can lead to microsatellite instability (MSI) that may affect cell growth and apoptosis mechanisms, both responsible for carcinogenic processes [Charames GS et al, 2003]. Lynch syndrome's affected patients have an increased risk (about 80%) to develop an early onset CRC, since the age of 42. The increased risk is also related to other neoplasms not strictly

correlated to colon. MSI and MMR mutations as well are not only involved in HNPCC: about 15% of non-hereditary CRC presents these genetic alterations, which are caused by a secondary modification, like aberrant methylation of MMR genes' promoters that causes an under-expression of the implicated genes. Lynch syndrome's identification criteria are:

- Familiar history: an early evaluation with Bethesda and Amsterdam criteria, that may establish if it is worth to proceed with further genetic analysis for HNPCC;
- Tumor-based test: MSI and immunostaining evaluation
- Foresight models: MMRpredict, MMRpro, PREMM [Vasen HF et al, 1991; Vasen HF et al, 1999; Umar A et al, 2004].

If these tests give a positive response, it is possible to proceed with the DNA isolation from blood cells. Further, the DNA can be sequenced to search specific mutations in MMR genes.

The Familiar Adenomatose Polyposis (FAP) FAP is the second major type of hereditary CRC syndrome, which accounts for less than 1% of all CRC cases [Wennstrom J et al, 1974]. Patients with FAP tend to develop numerous adenomas at a younger age that, within 40 years of age, undergo malignant transformation, unless colon or rectum is not removed. This disease is inherited with an autosomal dominant model, with a penetrance of 100%. It is caused by a germline mutation in the tumor suppressor gene APC; the majority of APC mutations are frameshift or nonsense mutations that lead to the synthesis of a truncated protein. Furthermore, somatic mutation of the APC gene has been identified in 80% of sporadic CRC as well as in some cancer of the stomach, pancreas, thyroid, ovary [Brown SL et al, 2007]. APC is involved in various cellular processes related to cell migration, cell adhesion, proliferation, differentiation, and chromosome segregation. Regarding CRC pathogenesis, it plays a central role in the negative regulation of the Wnt signaling pathway which controls cell proliferation and differentiation in the gastrointestinal tract. APC is a component of the Axin-APC degradosome complex that promotes the ubiquitin-dependent proteasomal degradation of the WNT effector β-catenin. Loss of function of APC leads to excess β-catenin accumulation within the

cytoplasm and translocation into the nucleus where it operates transcriptional switch leading to activation of ciclin D1, MYC and many other genes, which are important drivers in tumor formation due to their roles in cell proliferation, apoptosis, and cell-cycle progression.

The familial forms of colorectal cancers are more common than hereditary forms but the genetic mechanisms underlying their origin are not clear. The patients with family history of CRC cases and in absence of clear genotype-phenotype correlation and germline mutation, are classified as familial CRC cases. Recently studies identified germline mutations, potentially involved in familial colorectal cancer type X; these mutations in the RSP20, SEMA4A, HNRNPAO, WIFI genes contribute to the regulation of PI3k and MAPK/ERK signaling. Familial CRC is probably caused by low penetrance mutations and polymorphisms influenced by genetics and environment complex interactions.

- IBD: Patients with inflammatory bowel disease (IBD) have a significantly increased risk of colorectal cancer because of the chronic inflammatory state insults to the intestinal mucosa. Tissue samples from patients with IBD-related colitis demonstrate increased expression of nitric oxygen synthase, increasing the local abundance of reactive oxygen species. This oxidative stress is associated with p53 loss of function mutations, hypermethylation of the MLH1 gene, and microsatellite instability

- Diabetes mellitus: hyperinsulinemia (involved in second type diabetes mellitus, insulin resistance and glucidic altered tolerance) seems to be related to CRC developing. Insulin is a growth factor that causes proliferation in colon mucous membrane. A meta-analysis between 14 studies estimated that patients with diabetes present an increased risk (20%) to develop rectal cancer.

1.3.3 Protective factors

The delivery of acetyl-salicylic acid and other NSAID has been recently correlated to polyp regression in patients affected by FAP. What makes this mechanism protective is that NSAID inhibits COX-2, which is over-expressed in 90% of CRC. Cyclooxygenase-2 is responsible for PGE_2 synthesis, which is involved in epithelial proliferation, following colon's mucous membrane

damages. A study has shown that aspirin is also involved in relapsing's risk reduction of patients with previous cancers and colorectal tumors.

1.3.4 Altered signaling pathways: genetic and epigenetic modifications

The main altered molecular pathway involved in the onset of colorectal cancer is the one involving APC/B-catenin and the mismatch repair (MMR) machinery which harbors gene mutations both in sporadic and in hereditary CRC.

Among the most commonly known gene alterations for CRC, there are mutations affecting the KRAS or BRAF genes; the alterations in these genes lead to hyperactivation of the MAP Kinase pathway. Furthermore, EphB2 can be downregulated by genomic loss or promoter methylation, also resulting in MAPK hyperactivation

KRAS is a proto-oncogene that is a downstream effector of the epidermal growth factor receptor. It signals through BRAF to activate the MAPK pathway. Mutations in KRAS or BRAF occur in approximately 55–60% of colorectal cancer, aberrantly activating the MAPK signaling pathway, inducing proliferation and suppressing apoptosis [Yamakuchi M et al, 2008; Lu H et al, 2010]. The most common signaling pathways that carry mutant genes in colorectal cancer include not only the RAS-RAF-MAPK pathway, but also the PI3K pathway, and TGFB1- SMAD pathway [Yamakuchi M et al, 2008].

In addition to genetic alterations, various epigenetic alterations are associated with the development of CRC including DNA methylation and the altered expression of non-coding RNA such as microRNAs (miRNAs).

DNA methylation refers to the enzymatic addition of a methyl group to the 5-position of cytosine by DNA methyltransferases (DNMT) to produce 5-methylcytosine. Generally, the favored substrate for DNMT is a CG dinucleotide sequence, named CpG. The majority of CpGs are methylated in normal mammalian cells with unmethylated CpGs being typically present only in regions of DNA called CpG Islands. Methylation of CpG islands within the promoter region is correlated with transcriptional silencing. DNA methylation is a normal mechanism in the mammalian genome by which cells regulate gene

expression, for example, DNA methylation is a major mechanism for X chromosome inactivation and genomic imprinting.

Several aberrant methylation phenomena have been highlighted in numerous genes in CRC tissue and cell lines [Zitta M et al, 2007].

Another important mechanism of epigenetic regulation is represented by miRNA expression. microRNAs (miRNAs) are a group of non-coding, small, single-single strand RNAs, composed by 21 to 23 nucleotides. They recognize a specific region of mRNA, which can be at either ends of it (5' and 3' UTR regions) or in the middle (Coding sequence), causing mRNA degradation [Ambros V et al, 2003; Perron MP et al, 2008], in order to negatively regulate gene expression at post-transcriptional level.

The function of many miRNAs remains currently unknown, but some of them seemed to have a role in pathophysiological processes, like tumorigenesis, apoptosis, cell survival and differentiation.

miRNAs share their function and final structure with another class of ssRNA called Small interfering RNAs (siRNA). In fact, both of them are constituted by about 20 nucleotides and have a role in a post-transcriptional level silencing with RISC complex [Ambros V et al, 2003; Kim VN et al, 2005; Kim VN et al, 2005]. What differentiates these two molecules is their origination modality: miRNAs come from an hairpin precursor made by 60 to 70 nucleotides, while siRNAs originate from double-stranded RNA [Bartel DP et al, 2004; Ambros V et al, 2003].

It is possible to find miRNAs in most eukaryotic cells, even in humans [Perron MP et al, 2008; Lee RCFR et al, 1993] where they represent about 1-5% of total genome and regulate almost 30% of coding genes [Rajewsky N, 2006; Stanczyk J et al, 2008].

1.4 Diagnosis and Staging

Screening is a secondary prevention method, which is useful to identify early cancerous damages, before clinical manifestation of the diseases. To perform an optimal screening, the pathology must follow certain guidelines, and CRC is a disease perfectly suitable for screening methods:

- High incidence and mortality tumor, that justifies high-cost screening campaign;
- Long lasting pre-clinic phase, in which is possible to implement an early intervention;
- High efficiency screening test for major sensitivity and specificity;
- A good therapy-beginning time may improve patients prognosis;
- Availability of non-invasive, simple and economic secondary prevention methods which the patients are willing to accept.

The importance of screening has been confirmed by a consistent decrease in CRC mortality rate in countries that adopted these methods. In USA a microsimulation analysis estimated that secondary prevention is responsible for a 53% mortality decrease [Edwards BK et al, 2010].

The most adopted screening methodology for the early diagnosis of colorectal cancer is based on fecal occult blood search. In Italy it is offered for free to people with > 50 years old every 2 years. [Howlader N et al, 2013]

The main limit of this test is the high number of false negative results. In fact, being negative to the test does not exclude the presence of CRC, because of the intermittent bleeding derived from mucous damages.

Another screening method is represented by endoscopic investigation, which is both a diagnostic and a therapeutic tool. Indeed, when a resectable polyp is found, it gives you the possibility to remove it, preventing the natural transition from adenoma to cancer [Winawer SJ et al, 1993]. High-risk patients undergo screening with more rigid timelines.

Lynch syndrome's CRC is very aggressive and can develop within 2 years from last negative colonoscopy [Vasen HF et al, 1995]. These patients undergo colonoscopy since the age of 25 or in 2 to 5 years from the age of the youngest relative with the earliest onset cancer. Screening frequency is about every two years up to 40 years of age, and after that age, every year. [National Comprehensive Cancer Network, 2015]

For FAP's affected patients, the screening campaign begins since puberty and ends with Gastro-duodenal endoscopy (GDE) at the age of 30. GDE is fundamental in order to exclude the presence of duodenal cancer, which is

accounted as the second common cause of death in FAP affected patients [Campos FG et al, 2015]. 95% of patients affected by FAP develop a duodenal adenomas. [Heiskanen I et al, 1999].
At present different diagnostic strategies are available.

1.4.1 Medical history

It is very important to have an overall view of patient's familiar history with CRC. It is useful to identify neoplasm-related risk factors and to correlate the eventual symptomatology with onset CRC, in combination with digital-anorectal investigation, that can put in evidence damages localized in low rectum up to 6 cm from the anal margin. Its dependence on the operator makes this analysis poorly sensitive, because of the difficulty distinguishing between inflammatory and neoplastic damages.

1.4.2 Instrumental analysis

Pancolonoscopy represents the most accurate diagnostic method with a sensibility of 97% and specificity of 98%. It can be conducted in the clinic under sedation. It is necessary to have a complete view of the colon, proceeding up to the caecum.
Different data in literature reported various risks correlated to this procedure: there's a risk of perforation of about 0,1%, a risk of hemorrhage that's about 0.3% and a mortality rate range that goes to 0.01% to 0.03% [Byers T et al, 1997].
In the last 50 years, proximal colon cancer has exceeded by frequency the distal colon cancer, with a substantial raise of localization in caecum [Mamazza J et al, 1982]. This variation is due to the improvement of prevention, diagnosis and treatment. In fact, colonoscopy is more accurate in distal colon than proximal colon, which tends to develop tight sessile adenomas, difficult to be seen with colonoscopy.

1.4.3 TNM Staging

TNM staging covers an important role in neoplasm evaluation, both in prognostic and therapeutic approaches. TNM is an acronym in which each letter

stands for a different factor taken in analysis: T stands for tumor extension, N indicates Lymph nodal chains and near lymph nodes involved, M stands for metastasis. Clinical TNM staging (cTNM) uses different diagnostic and instrumental methods to evaluate metastasis presence and spreading in other locations. T and N values before surgical operations, usually don't change the therapeutic approach, according to ESMO consensus guidelines for management of patients with colon and rectal cancer (ESMO Guidelines).

1.5 Treatment

Therapeutic approach must be planned based on different factors regard the patient:

- Carcino-embryo antigen evaluation (CEA): it is a tumoral marker that ensures important information about the patient's follow-up
- Age
- Intestinal occlusion presence;
- Symptoms duration;
- Disease location;
- Neoplasm stage;

It is important to involve the patient in treatment choices.

1.5.1 Pharmacological Treatments

The use of adjuvant pharmacological therapies depends on the stage of the disease:

- Stage I: T1-2, N0; there are no medical treatments expected after surgical intervention. The only thing to do is a patient follow-up
- Stage II: T3-4, N0, M0; the use of adjuvant therapy based on fluoropyrimidine and oxaliplatinum is not fully approved and depends on the patient's prognostic factors that increase the risk of relapsing.
- Stage III: T4, N1, M1; patients with stage 3 CRC are candidates for adjuvant chemotherapy, the use of which determines a 33% reduction in death risk and an increase of 10-15% in survival. Therapy's beginning is expected within 6 to 8 weeks since the surgery. Therapeutic scheme includes the

combination of 5-fluorouracil (5-FU) and folinic acid, which may be administered both orally and intravenous.

Capecitabine, a prodrug of 5-FU, is a valid and more tolerable alternative to the traditional one [Twelves C et al, 2005].

Actual therapeutic schemes expect the use of FOLFOX (combination of folinic acid, 5-FU and oxaliplatin) or, in alternative, XELOX (Xeloda=capecitabin and Oxaliplatin). Patients with good prognosis or intolerance to oxaliplatin receive a treatment with capecitabine or 5-FU + Folic Acids. These treatments have an optimal duration of 6 months [Mamazza J et al, 1982; Schmoll HJ et al; Van Cutsem E et al, 2008].

A tumor is defined as advanced when shows high extension or an increased metastatic behavior at diagnosis. In this case, it is not possible to perform several therapies.

In patients in which an advanced tumor is found at the time of diagnosis, therapeutic approach is finalized to:

- Curing the disease (in a small number of cases)
- Symptoms palliation;
- Increase of patients' overall survival;
- Quality life improvement;
- Disease progression slowdown;
- Neoplasm reduction;

Literature data suggest to investigate, prior to select a therapeutical approach, about RAS mutations (exons 2,3,4 of KRAS and NRAS) and BRAF to define an optimal scheme to cure metastatic disease [Douillard JU et al, 2013].

Fluoropyrimidine, Irinotecan, oxaliplatin, monoclonal anti-EGFR antibodies and small molecules that inhibit angiogenesis like regorafenib are all useful drugs in advanced disease treatment.

The gold standard in disease treatment is represented by FOLFOX/FOLFIRI In association with bevacizumab, an anti-VEGF monoclonal antibody, regardless the mutational state of KRAS and NRAS.

Several studies evidenced an increase in survival of patients that continue taking bevacizumab and an improvement in those in whom it has been re-administered after a 3-month interruption [Grothey A et al, 2008; Masi G et al, 2005].

The usage of anti-EGFR drugs requires a previous mutational investigation about KRAS, NRAS and BRAF. If gain of function (GOF) genetic alterations are present, EGFR-related pathways are over-activated and this could frustrate anti-EGFR therapy. Anti-EGFR drugs based on small molecules (like Cetuximab) can be used in patients with non-mutated (WT) RAS, regardless of treatment scheme, in association with FOLFOX/FOLFIRI or in monotherapy [Jonker DJ et al, 2007; Karapetis CS et al, 2008].

If patients treated with first line therapy don't show a disease regression, it is possible to switch to a second line therapy, which provides different drugs association chosen according to previous therapeutic schemes. E.g.: if a FOLFOX + bevacizumab therapy doesn't display good results (like tumor mass reduction), it is possible to try a new therapy line which is composed by FOLFIRI in association with Aflibercept [Van Cutsem E et al, 2012]. In patients in whom multiple standard cytotoxic drugs have been administered, it is possible to proceed with regorafenib delivery.

1.5.2 Surgical Treatments

A great number of surgical operations can be performed based to the location of the CRC

The major surgical procedures for the right colon include right hemicolectomy and extended right hemicolectomy (both performed open or with a laparoscopic approach).

An extended right hemicolectomy includes the transverse colon to the splenic flexure. This procedure includes the left branch of the middle colic artery. The procedure is appropriate for tumors at the hepatic flexure and in the transverse colon. Many surgeons avoid isolated transverse colon resections because a hepatic flexure to splenic flexure anastomosis is a potentially problematic one.

Right Colon

Right Hemicolectomy (Open)

The patient is placed on the operating table in supine position.

The peritoneal cavity is entered via a midline incision.

The abdomen is explored to determine the presence and extent of any metastatic disease.

An assessment of tumor resectability is made with special attention paid to the duodenum, pancreas, great vessels, and right kidney and ureter. This is mostly achieved by visual inspection, as the tumor should not be manipulated prior to vascular ligation.

The small bowel is first retracted to the patient's right side in order to expose the base of its mesentery.

The peritoneum overlying the base of the small bowel mesentery is then incised just above its border with the fourth portion of the duodenum and this incision is extended caudally for approximately 6 cm. Careful dissection is then undertaken in a plane posterior to the superior mesenteric vessels to separate the mesentery from the retroperitoneum. This dissection proceeds in a medial-to-lateral direction until the surgeon can insert the second and third fingers of his nondominant hand behind the superior mesenteric vessels and the fingertips come to lie on either side of the ileocolic vascular pedicle (Figure 1).

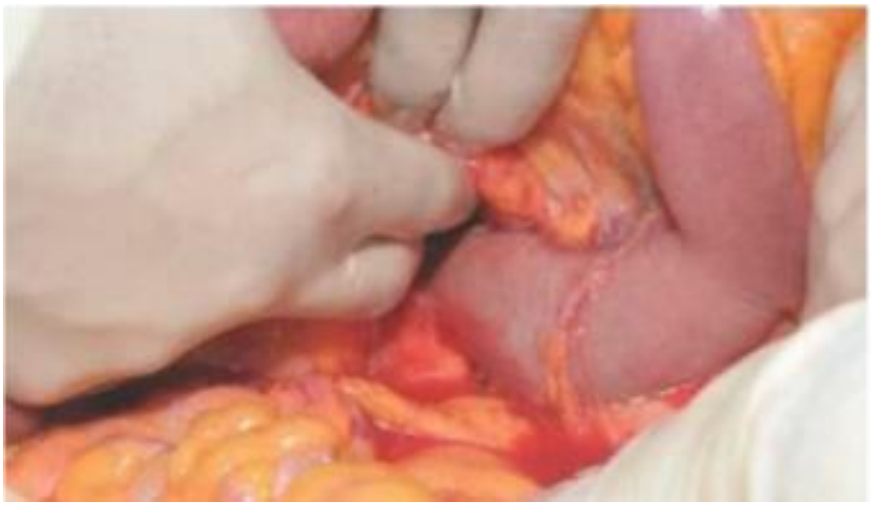

Figure 1. Hand of the surgeon behind the superior mesenteric vessels

The small bowel and its mesentery are then reflected back to the patient's left to set up vascular division.

Mesenteric windows are then opened on either side of the ileocolic pedicle near its origin using the fingertips of the surgeon's nondominant hand as a guide (Figure 2).

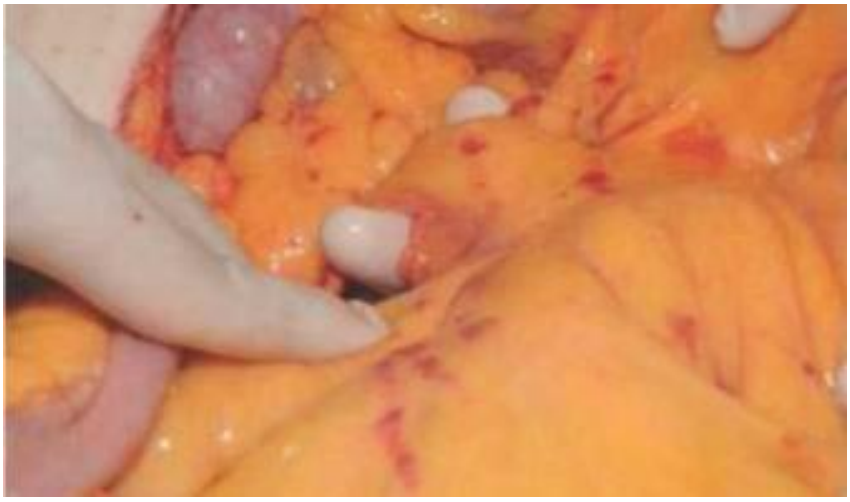

Figure 2. Mesenteric window on the ileocolic pedicle.

After clearing lymphatic tissue from the vessel origins, clamps are applied and the vessels are divided and ligated.

Through this window in the mesentery, the plane between the ascending mesocolon, and the retroperitoneal structures is developed in a cephalad direction.

The surgeon will encounter the most medial aspect of Gerota's fascia and the anterior surface of the duodenum and pancreatic head during this part of the operation. As dissection proceeds above the duodenum, a plane between the first portion of the duodenum and the transverse mesocolon will be entered.

At this point the right branch of the middle colic artery and vein are mobilized to their origin.

The right branch of the middle colic artery and vein are then ligated and divided. The remaining mesentery adjacent to the mid-transverse colon, which includes the marginal artery, is then divided. Pulsatile arterial bleeding should be confirmed from the distal end of the divided marginal artery prior to ligation. This indicates adequate blood supply on the colon side for creation of the ileocolic anastomosis.

The remaining mesentery adjacent to the terminal ileum containing the two marginal ileal vessels is also divided.

With the lymphovascular drainage of the right colon now interrupted, the tumor may be manipulated without fear of disseminating malignant cells. Attention is turned to the omentum and its attachments to the transverse colon.

Beginning at the anticipated site of division in the mid-transverse colon, the omentum is separated from the transverse colon itself and the transverse mesocolon by developing the avascular plane. This dissection proceeds from the mid-transverse colon toward the hepatic flexure. A "crossing" vein between the omentum and the proximal transverse mesocolon is often encountered at this point in the operation.

After this vein has been divided and ligated the omentum should be completely free from the proximal half of the transverse colon and its mesocolon and the lesser sac fully exposed.

The lateral attachments of the right colon are carefully mobilized and the hepatic flexure attachments divided, taking care to identify the right ureter during the course of this dissection. Precise dissection at the junction of the pericolic fat and lateral areolar tissue, rather than in the middle of the areolar tissue plane itself, will minimize risk of injury to the ureter.

The mid-transverse colon and the terminal ileum are then divided between clamps and the right colectomy is performed.

Ileocolic anastomosis is performed according to the surgeon's preference.

The mesentery of the ileum and transverse colon are reapproximated with a running absorbable suture and the omentum is placed over the anastomosis. Incorporating the tip of the omentum into the tie at the end of the mesentery closure suture line will ensure that it stays in place over the anastomosis.

Laparoscopic right hemicolectomy

Two general approaches are possible in laparoscopi right hemicolectomy, one where the colon is mobilized from its lateral attachment first (the lateral approach), and one where the vascular pediclesare initially ligated, followed by colonic mobilization (the medial approach). Both accomplish the same

dissection, but advantages to the medial-to-lateral approach include the following: early ligation of the vascular pedicles in cancer may theoretically prevent the liberation of tumor cells into the mesenteric circulation during mobilization.

Preservation of the lateral colonic ligament until the end of the mobilization keeps the right colon fixed in place, limiting the need to manipulate a floppy colon.

There are both absolute and relative contraindications to the laparoscopic approach to colectomy.

Absolute contraindications include: Hemodynamic instability, known history of extensive adhesions from prior surgery.

The relative contraindications to laparoscopy depend on each clinical circumstance, and the skills and comfort levels of the surgeon, including

large tumor size (>8 cm); tumor invading other structures; bowel dilation from obstruction or ileus; emergency surgery; history of prior surgery.

A patient may have had many operations in the past, but the amount of adhesions may not prohibit a subsequent laparoscopic colectomy.

When extensive adhesions are present and a conversion to open surgery is necessary, it is important that the decision to convert is made early in the operation.

The camera port is placed in a periumbilical position.

In the majority of cases, this periumbilical port wound is extended around the umbilicus for exteriorization of the colon, resection, and anastomosis.

The surgeon begins the operation from the left side of the patient using the

left lower quadrant and suprapubic ports. The assistant stands to the right of the surgeon, holding the camera and using the left upper port.

A monitor near the right shoulder of the patient is used by both operators. After vascular ligation and medial-to-lateral retromesenteric dissection, the surgeon moves to the right of the assistant, using the two left-sided ports, for the hepatic flexure takedown and the mobilization of the lateral ligament.

The assistant helps through the suprapubic port.

The patient is placed in a slight Trendelenburg position.

The omentum is lifted above the transverse colon, and the distal ileum is moved into the pelvis. The patient is tilted steeply with the right side up, and the small bowel loops are swept to the left of the midline.

The operation starts with the isolation of the ileocolic pedicle.

The ileocolic artery is a proximal branch of the superior mesenteric artery that courses just inferior to the third portion of the duodenum.

Therefore, the identification of the duodenal sweep through the mesentery when the transverse colon is superiorly retracted is an important initial step in identifying the ileocolic pedicle. Tension on this vessel is critical in distinguishing it from the superior mesenteric vessels.

The right colic artery arises from the ileocolic pedicle to supply the hepatic flexure about 90% of the time, and does not need to be ligated separately.

Distal in its course, near the ileocecal junction, the ileocolic artery becomes the ileal branch (and accessory ileal branch), which can bleed if injured. Therefore, the dissection of the ileocolic artery should start in the avascular plane between the superior mesenteric vessels and the ileal branch (Figure 3).

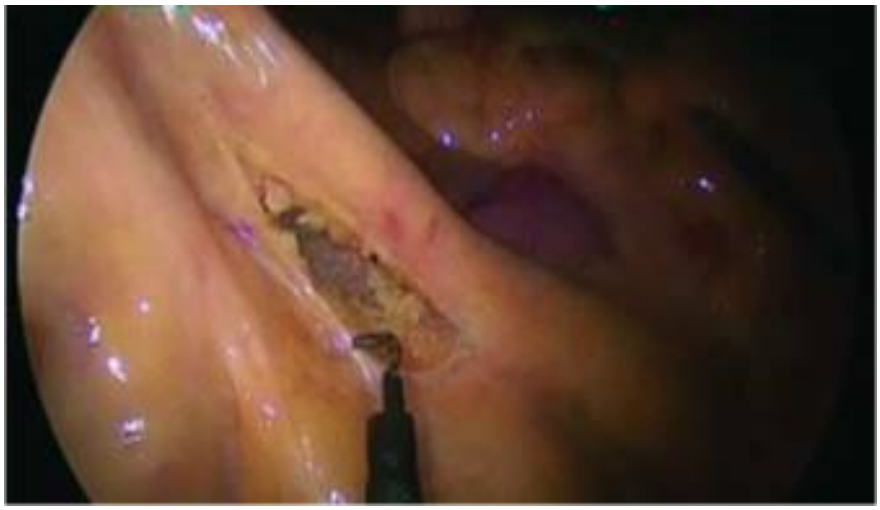

Figure 3. Avascular plane between the superior mesenteric vessels and the ileal branch.

A wide window is made in the peritoneum inferior to the ileocolic pedicle as the retroperitoneal structures are gently swept away in a posterior direction. A mesenteric window is then made on the superior aspect of the ileocolic pedicle, and the pedicle should be isolated adequately to allow an easy vessel division. The surgeon should clearly identify the duodenum to avoid injury.

The division of the ileocolic pedicle can be performed using vessel sealing energy devices, laparoscopic staplers, or clips.

The next series of maneuvers will assist in the identification of the middle colic vessels. First, the previously cut leaf of the peritoneum overlying the duodenum is lifted.

The duodenum and head of pancreas are then swept posteriorly and separated from the right side of the middle colic vessels. This step must be performed carefully and gently, as excessive force will cause a rip in the pancreaticoduodenal or gastroepiploic vein, resulting in significant hemorrhage. This dissection proceeds deeper and in a cephalad direction, until the transverse colon is separated from the duodenum.

Once there is adequate space to the right of the middle colic vessels, the middle colic pedicle is anteriorly lifted using two points of retraction, one to the right and one to the left of the pedicle. This maneuver is critical in the identification of the right and left branches of the middle colic vessels. The goal of the procedure is to divide the right branch of the middle colic vessels to harvest the lymph nodes draining the hepatic flexure and proximal transverse colon. The middle colic artery supplies the transverse colon and arises from the superior mesenteric artery at the inferior base of the pancreas. There may be one, two, or three branches off the superior mesenteric artery, and the classic Y-shaped single trunk occurs in less than 50% of cases. An imaginary line is created from the base of the middle colic vessels toward the anticipated transection point of the transverse colon.

The peritoneum of the transverse mesocolon is then divided along this line. The takeoff of the right branch is then identified and divided at its origin. In addition to the middle colic vessels, one will encounter a vein from the head of the pancreas to the hepatic flexure (the right colic vein, located just to the right of the middle colic vessels). This vein is isolated and divided, taking care not to injure the right gastroepiploic vein, which is its adjacent branch running on the surface of the pancreas toward the stomach.

The right colon mesentery is then separated from the retroperitoneum in a medial-to-lateral direction. With the cut edge of the right colon mesentery

retracted anteriorly, the retroperitoneal fascia, or white line of Toldt, is identified at its medial aspect, and bluntly separated from the mesentery. This is essentially avascular, and this retromesenteric dissection is taken underneath the hepatic flexure and the ascending colon to the lateral abdominal wall. This dissection should not be carried too far posteriorly, into or underneath Gerota's fascia, and following the retroperitoneal plane of the duodenum more laterally will help maintain this proper plane. At this stage, the hepatic flexure and a thin lateral ligament of the ascending colon act as a natural retractor, keeping the otherwise floppy right colon in place.

At the level of the falciform ligament, the gastrocolic ligament is opened. As the transverse colon is inferiorly retracted, the lesser sac is dissected, and the congenital adhesions of the posterior omental leaf and the transverse mesocolon are undone. Adequate traction and tissue triangulation are necessary to identify the correct plane of dissection. Avoiding injury to the right gastroepiploic vessels, the previously dissected retromesenteric plane from the medial approach is then identified. With the transverse colon inferiorly retracted, from left to right, the hepatic flexure is taken down.

The lateral ligament of the ascending colon is divided from superiorly as the dissected colon is gradually retracted into the pelvis, until the right psoas muscle and right iliac vessels are identified. The retroperitoneal fascia is preserved, as the mesentery of the ileocecal region is widely dissected from the retroperitoneum. It is often possible to identify the right ureter during this dissection, and this structure should be maintained underneath the intact retroperitoneal fascia if the dissection is properly performed.

The only remaining attachments are from the ileum to the retroperitoneum. The patient is now placed in the steep Trendelenburg position as the dissected right colon is placed back into its original position. The small bowel loops in the pelvis are completely retracted in a superior direction.

With the distal ileum retracted anteriorly and superiorly, the ileal attachments to the retroperitoneum are taken down. Strong traction is needed to retract the tissues away from the right iliac vessels and to avoid injury to the right ureter. This dissection is taken laterally around the appendix and cecum, meeting the

previous superior dissection. The medial extent of this ileal mobilization is the right iliac vessel; this will ensure adequate reach of the small bowel to the transverse colon for anastomosis.

At this point in time, the intracorporeal dissection is complete, and the right colon is ready for exteriorization, bowel transection, and extracorporeal anastomosis.

A small incision is now created. Prior to making the incision, however, one must ensure adequate reach of the transverse colon to the proposed incision site; if not, one risks an unnecessarily difficult anastomosis, or undue tension and tearing of the middle colic vessels.

A wound retractor is placed to avoid a port site recurrence in cases of malignancy. The grasped ileocecal region is brought into view through the small incision, and the dissected right colon is exteriorized and placed in its native configuration. The remainder of the ileal mesentery and marginal artery of the transverse colon are dissected toward the bowel wall. The bowel is
divided and an ileocolic anastomosis is created. The type of anastomosis depends on surgeon preference (hand-sewn, stapled functional end-to end, or stapled end-to-side anastomosis).

Left Colon

Left Hemicolectomy (open)

The patient is positioned in lithotomy position to allow access trough the anal canal of a surgical stapler, and to let the surgeon or an assistant to stand between the patient's legs during periods of difficult dissection.

The preferred incision for laparotomy is the midline as it allows access to the entire abdominal cavity.

Inspection and palpation of all internal organs is done with special attention to the liver lobes for metastatic disease; the nasogastric tube placement is confirmed. The peritoneal surface of the abdominal cavity and pelvic floor and organs are also inspected.

With an assistant retracting the colon laterally, an abdominal pad is placed wide

open to encircle the small intestines and position them in the right upper quadrant of the abdomen.

This maneuver allows full exposure of the left colon, sigmoid, and upper rectum. The inferior mesenteric artery (IMA) will be located on the anterior surface of the aorta below the third portion of the duodenum.

The peritoneum is incised with the electrocautery at the sacral promontory on the right side with left and outwards retraction of the rectosigmoid junction.

The incision is extended to the ligament of Treitz.

In most patients, this maneuver will expose the areolar plane over the presacral area and anterior to the aorta and common iliac arteries. The vascular pedicle is easily identified and cranial dissection is performed with electrocautery to the IMA origin. Lateral structures at the level of the promontory are recognized (including the left iliac artery, gonadal vessel, and left ureter) (Figure 4).

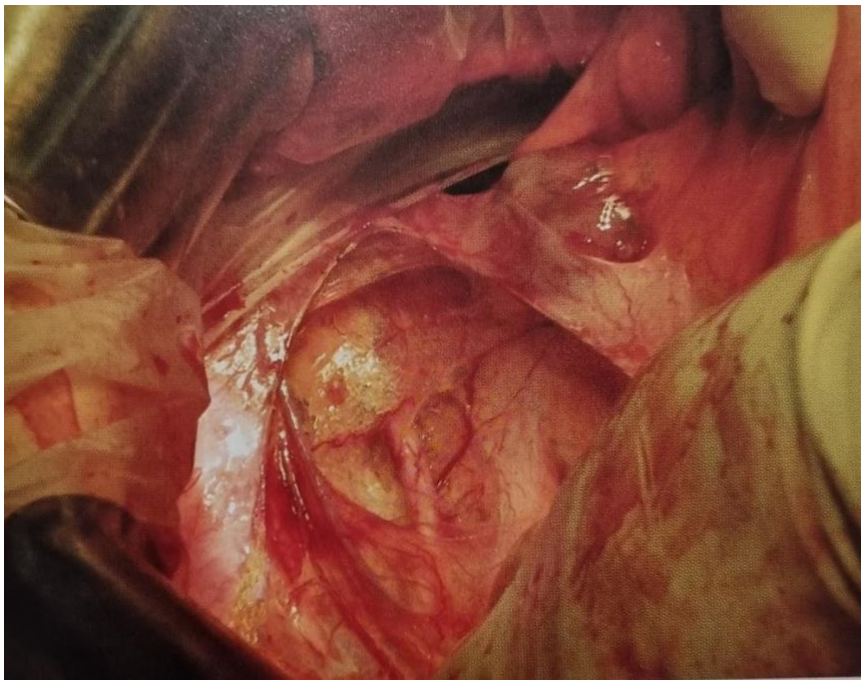

Figure 4. Lateral structures at the level of the promontory.

These structures can be tracked upwards and left intact in the retroperitoneum, thus avoiding injury during the ligation of the vascular pedicles. The bifurcation of the parasympathetic plexus is identified and is preserved.

The pedicle of the IMA is then divided with a sealing device proximal to the origin of the left colic vessel.

The entire lymphovascular pedicle allows proper staging. The anatomic planes are followed in a cephalad direction until the inferior mesenteric vein (IMV) is identified, and then isolated and divided.

At that point, the entire medial aspect of the colon has been freed and the lateral dissection is performed above the Gerota's fascia, the tail of the pancreas, and the tip of the spleen. The dissection is extended laterally to the paracolic gutter.

Lateral incision of the line of Toldt allows full mobilization of the left segment of the colon to the level of the splenic flexure.

The distal transverse colon is mobilized from the omentum, through the gastrocolic omentum if necessary, to enter the lesser sac and expose the posterior surface of the stomach.

The lateral dissection of the areolar plane is performed with the electrocautery or the vascular sealing devices since there may be a middle size vessel involved in the planes.

The posterior areolar plane is developed all the way to the lateral attachment of the line of Toldt.

The splenic flexure is then peeled, medial to lateral, from the tail of the pancreas, Gerota's fascia, and retroperitoneal structures.

Care must be taken to avoid excessive traction that may injure the marginal artery of Riolano in the mesentery of the splenic flexure that will be needed to supply the portion of colon intended for anastomosis or colostomy.

Once the transverse colon is mobilized, the left branch of the middle colic artery is taken at its origin, which normally allows full mobilization of the transected colon to the level of the pelvis.

The distal left colon or sigmoid or rectum can be divided with a transverse stapler at the desired level appropriate for the disease process. The mesenteric vessels can be sealed or ligated as the mesentery is divided at right angles to the point of bowel transection.

The stapler is passed through the anus and guided up to the stapled end of the colon or rectum (Figure 5).

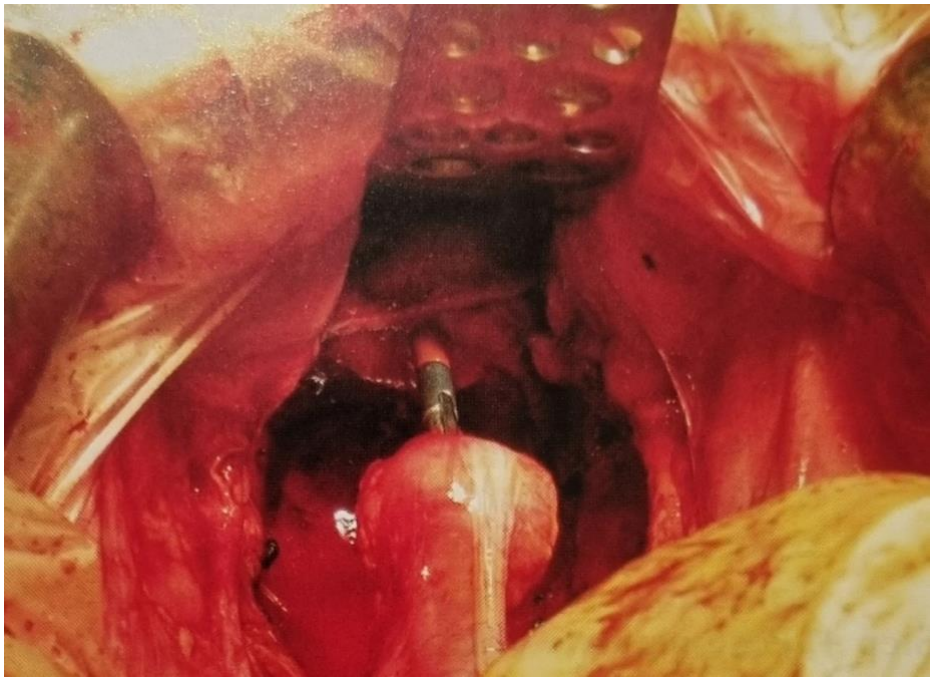

Figure 5. End-to-end anastomosis.

After firing the stapler, the anastomosis is inspected: air is insufflated with the proximal bowel occluded, and the anastomosis submerged in saline solution to inspect for bubbling—the so-called "leak test."

In the event of a positive test, the anastomosis can be reinforced with sutures or occasionally may need to be resected and re-performed.

Another alternative is to perform a hand-sewn anastomosis.

Laparoscopic left hemicolectomy

The patient is placed in the modified lithotomy position

The monitors should be positioned so that they are available near the left shoulder of the patient.

The primary insufflation site is placed off of the midline, generally two fingers lateral to and above the umbilicus in the right upper quadrant. Once insufflation is obtained, a 5 mm trocar is placed at that site and then two additional trocars are placed, one 5 mm size in the epigastric area just slightly to the left of midline and a 12 mm trocar in the right lower quadrant just medial and slightly superior to the anterior superior iliac spine. If necessary, a fourth 5 mm trocar can be placed in the left lower quadrant lateral to the rectus muscle, and this fourth

trocar site can then be utilized for a muscle splitting incision for extraction of the specimen and insertion of the circular stapling anvil if utilized.

To begin the left colon mobilization, the patient is placed in steep Trendelenburg with slight left side up tilt and the small intestine is mobilized out of the pelvis. At this time any attachments of the sigmoid colon to the left pelvis or lateral pelvic side wall are appreciated.

The mesosigmoid is grasped near its mid to distal portion using an atraumatic grasper and the colon is allowed, if mobile, to flip behind the mesentery out of the view of the operating surgeon.

A grasper, placed through the epigastric trocar elevates the mesosigmoid anteriorly towards the anterior abdominal wall. It can be moved slightly from right to left to identify the plane of dissection behind the mesocolon just at or above the sacral promontory. The iliac vessels are often visualized at this time through the retroperitoneal surface and in a thin patient the right ureter may also be obvious.

Dissection is started in this plane behind the mesosigmoid and above the sacral promontory but caudal to the inferior mesenteric artery (IMA) origin. The IMA is usually obvious because when the mesosigmoid is grasped and elevated anteriorly, it typically tents up and is quite prominent as the dissection continues. The peritoneum overlying the dissection plane is scored along the sacral promontory into the pelvis and also cephalad toward the IMA. Establishing this dissection plane is an essential first step.

Dissection should be anterior to the iliac vessels and to the hypogastric nerves, which are typically easily seen through this plane. Once this dissection plane is established, blunt dissection using an atraumatic instrument is often possible to begin the dissection and to establish this tissue plane. The left ureter should now be identified, and the dissection plane, once the posterior aspect of the mesocolon is reached, should actually be in an anterior angled direction.

Once the ureter is identified and traced, dissection is continued on the peritoneal surface, scoring and dissecting out the IMA and vein. The clear area cephalad toward the IMA and vein within the mesentery of the mesosigmoid is identified and the vessels themselves are then freely mobile off the retroperitoneum. The

artery is then divided either utilizing a bipolar energy device or other techniques including clips and/or staples.

The vein can be divided at the same time or individually at this same location, or in the case of a planned low anastomosis, the vein may be preferentially divided at the level of the pancreas at a later time.

The IMA division allows free mobility of the mesosigmoid off the retroperitoneum so that dissection can continue in this medial-lateral plane all the way to the posterior-lateral edge of the sigmoid and then up behind the descending colon.

The dissection continues in this plane both cranially and caudally deep into the pelvis. Upon completion of this dissection, the colon is then grasped on its medial aspect and lateral incision of the peritoneal attachments is commenced usually using sharp dissection technique.

Lateral incision is continued both cranially including the splenic flexure and caudally down into the true pelvis. The proximal line of resection is then selected based largely on the mesenteric blood supply and location of pathology, but may also be determined by the quality of the sigmoid colon and the presence or absence of previous radiation therapy.

This obviously may affect the degree of splenic flexure mobilization necessary to result in a tension-free anastomosis.

Splenic flexure mobilization begins by putting the patient into a slight reverse Trendelenburg position with left side elevated. The omentum is grasped and elevated cranially to identify the transverse colon and then the splenic flexure is mobilized either by a continuation of the lateral approach as would commonly be done with an open operation, or with a medial-lateral approach starting from the lesser sac entered by a tissue plane identified between the omentum and the transverse colon and then extended over laterally from that direction.

Once the splenic flexure is fully mobilized, the proximal site of planned resection is grasped and brought down into the pelvis to insure that there is adequate mobility for a tension-free anastomosis at the distal planned line of resection.

If mobility is not adequate, it may require further division of the inferior mesenteric vein at the level of the pancreas, if not already conducted.

At this point the patient is placed back into Trendelenburg position and the distal line of resection is then chosen, either based on anatomic landmarks or on endoscopic confirmation in the case of a neoplasm. Tattooing is of some value but cannot be fully relied on because of the nonspecificity of the exact location when dealing with rectal neoplasms and anticipated margins of 2 cm or even less.

Rectum

Low anterior resection (Open)

For the greater part of the early to mid-20th century, abdominoperineal resection with permanent colostomy was the mainstay surgical option for patients with rectal cancer. With the advent of surgical staplers and anastomotic techniques for low pelvic anastomoses, sphincter preservation surgery became the preferred option for the majority of rectal tumors.

Low anterior resection with restorative intent is possible for tumors in the distal third of the rectum that do not invade the sphincter musculature.

The surgery is approached via a midline incision. Upon entering the abdomen, a thorough exploration is performed to exclude metastatic disease. The sigmoid and descending colon are mobilized medially and the left ureter is identified. An assessment is made about the length of the descending and sigmoid colon, and the need for splenic flexure mobilization. The peritoneum on both sides of the rectum is incised at the level of the sacral promontory, with care to avoid injury to the ureters and to the sympathetic nerves.

The dissection is carried underneath the superior rectal artery, and the superior rectal artery is dissected to the level of the left colic artery and inferior mesenteric artery. The decision of the location of vessel ligation is based on the need for adequate length for a tension free and well-vascularized anastomosis.

Division at a level just inferior to the left colic artery, with preservation of the left colic artery will result in more predictable blood supply to the anastomosis, but may not give sufficient length, especially in cases where the majority of the sigmoid is resected.

Division at the level of the inferior mesenteric artery, at its takeoff from the aorta, along with proximal division of the inferior mesenteric vein, will typically ensure sufficient length for the anastomosis.

The anastomosis will then rely on blood supply from the marginal artery of Riolano, based on the middle colic artery.

The purpose of an anterior resection for cancers of the mid and low rectum is complete removal of the lymph node bearing mesorectum along with its intact enveloping fascia, a technique referred to as total mesorectal excision (TME).

The mesorectum is enveloped by the fascia propria (11). The proper plane of dissection is initiated by following the posterior aspect of the superior rectal artery until a shiny, filmy membrane is encountered at the pelvic brim. This plane lies between the fascia propria of the rectum containing the mesorectum and its vessels and lymph nodes, and the endopelvic fascia, which covers the hypogastric nerves and pelvic plexuses. The dissection proceeds posteriorly along this plane, keeping in mind that the fascia propria may be tethered to the presacral fascia at the level of the fourth sacral vertebra, sometimes referred to as the rectosacral fascia or ligament. At this point, it is important to avoid entering the presacral fascia for fear of injuring the presacral veins, which may result in significant bleeding.

The dissection is carried posteriorly as far as can be accomplished safely under direct

vision. The surgeon should avoid the technique of blind blunt dissection as this technique may result in breach of the fascia propria and an incomplete mesorectal excision. The anterolateral dissection is initiated by incising the peritoneum in the pouch of Douglas and dividing the remaining peritoneum laterally, avoiding injury to the pelvic sidewall and its vessels.

The anterior dissection is carried out in front of Denonvillier's fascia, which lies posterior to the prostate and seminal vesicles in males and the vault of the vagina in females, and anterior to the extraperitoneal rectum, anterior mesorectum, and fascia propria.

It is important to recognize that immediately anterior to Denonvillier's fascia

lie the parasympathetic nerves that supply the corpora and erectile function in males. These small nerves are in very close proximity during this anterior dissection and are in jeopardy of injury.

The posterior dissection is carried out below the rectosacral fascia down to the level of the retrorectal space, whose inferior portion corresponds to Waldeyer's fascia. The entire mesorectum is thereby contained within the specimen.

The location of the point of transection is determined in part by the location of the tumor. A distal resection margin of 1 cm is now considered adequate for oncological outcome.

The traditional end-to-end coloanal anastomosis was the default technique for many years. However, a constellation of symptoms attributed to the loss of reservoir, including urgency, clustering of evacuations, and incontinence, has prompted surgeons to seek alternative anastomotic techniques. It has been proposed the creation of a pouch with a side-to-endo anastomosis.

Either technique is acceptable, keeping in mind that the size of the pouch or the length of the defunctionalized limb should not exceed 6 cm.

The anastomosis is created with the circular stapler, using the double-stapled technique. For cases that involved a mucosectomy, a hand-sewn anastomosis is preferred.

The anastomotic integrity is tested.

Laparoscopic low anterior resection

The incision is made above or below the umbilicus.

An extension of this incision may be used for specimen extraction, thereby providing somewhat better cosmesis. The Hasson technique starts with a vertical 1.5-cm long skin incision, dissecting the subcutaneous tissues to the level of fascia.

The fascia is grasped with Kocher clamps or similar instrument to better visualize and incise that layer. Anchoring sutures are placed on the edges of the fascial incision with 2-0 Vicryl™ (Ethicon Inc., Summerville, NJ) or silk to form handles for the Hasson trocar. The

preperitoneal fat is gently spread to expose the peritoneum, which is grasped with smaller clamps and divided, taking care to ensure that there is no intervening bowel. The 12-mm Hasson trocar is then introduced into the abdominal cavity and secured with the previously placed anchor sutures.

The camera is then introduced into the abdominal cavity. A careful survey of the entire abdominal cavity is performed to note any adhesions and any relevant disease of the liver or peritoneal surfaces (to exclude tumor metastasis).

Placement of the ports should be done judiciously to allow for adequate triangulation of the instruments and freedom of motion. The right lower quadrant port should be about 2–3 cm medial and superior to anterior superior iliac spine, while the right upper quadrant port should be a handsbreadth above this, allowing a centimeter or two margin from the lower ribs.

A left lower quadrant or suprapubic trocar may be useful for some cases.

Using 10–12 mm ports for all port sites provides flexibility for the angle of the camera and stapler.

After the introduction of the ports, the patient is turned to right-side-down position with steep Trendelenburg. This facilitates displacement of the omentum and small-bowel loops toward the right side of the abdominal cavity.

The patient is turned with the left side up so that the small bowel can be swept away from the root of the mesentery. The IMA is identified and skeletonized, then divided with vascular staples or an energy source.

The bare area of the mesentery is divided caudally, which brings the plane of dissection directly to the IMV as it is exposed just lateral to the duodenum. The IMV is divided in a similar manner to the IMA. The colonic mesentery is dissected bluntly away from the retroperitoneum, using the ureter as a guide for the intervening plane.

This dissection is continued proximally and distally until the only remaining attachments are the lateral most attachments to the abdominal sidewall, spleen and omentum.

The patient is brought back to a steep Trendelenburg position to allow the small bowel and omentum to fall away from the operative field. The camera is oriented toward the pelvis and dissection is continued posteriorly while preserving the

hypogastric nerves. Mobilization of that plane ultimately leads to the avascular plane between the mesorectum and the presacral fascia.

The first assistant should provide adequate traction of the proximal rectum superiorly and laterally. The posterior dissection is continued distally and on either side to include the division of the lateral rectal stalks.

Lastly, the anterior dissection is performed to complete the total mesorectal excision (TME).

Denonvillier's fascia in male patients is swept anteriorly, separating the seminal vesicles from the mesorectum.

The principles of a total mesorectal excision should be followed down to 1–2 cm distal to the tumor, but the mesorectal envelope should be mobilized to 5 cm below the tumor.

1.6 microRNAs

microRNAs or miRNAs are a group of small endogenous single-stranded non-coding RNAs that have a post-transcriptional regulatory function. miRNAs were first observed by Victor Ambros, Rosalind Lee and Rhonda Feinbaum in the early 1990s during a study on the larval development of Caenorhabditis elegans [Lu H et al, 2010]. In the following years, these molecules have been reported in a wide variety of organisms, from single-cell algae to humans, suggesting that miRNA-mediated biological function is an ancient and critical cellular regulatory system [Wang J et al, 2019; Weiner AMJ, 2018]. The importance of miRNA activity is further suggested by the high conservation in evolution of both miRNA genes and processing machinery. miRNA genes are located within the introns or exons of protein-coding genes, as well as in intergenic areas. In addition, the number of miRNAs in the genome appears to be correlated with the complexity of organisms with mammals having the largest number of miRNAs [Lu Y et al, 2018]. The biogenesis of these non-coding RNA starts similarly to protein-coding transcripts with a primary transcript RNA (pri-miRNA), transcribed by RNA polymerase II, that is modified with the addition of a 5′ cap and a 3′ poly-A tail. Then, the primary transcript is processed into a small stem-loop structure of 55-70 nt by Drosha, a specific ribonuclease, in association with

DGCR8. The precursor miRNA (pre-miRNA) is ten exported to the cytoplasm by exportin-5, which is a member of the Ran-dependent nuclear transport receptor family. In the cytoplasm pre-miRNA hairpin are cleaved by Dicer, another specific ribonuclease, with the help of TRBP (transactivating response RNA-binding protein) to produce mature miRNA duplex 19-22 nt in length [LaPierre MP et al, 2017; Kotyla et al, 2020; Rupaimoole R et al, 2016; Cowden Dahl KD et al, 2009]. miRNA is loaded onto AGO2 (Argonaut 2 protein) and RISC (RNA-induced silencing complex). Generally, only one strand, termed guide strand, is associated with the silencing complex and the other one is degraded; however, both strands can act independently on different targets. The silencing complex performs its regulatory action using one of 2 post-transcriptional mechanisms: mRNA cleavage or translational repression. The miRNA presents a sequence called seed sequence that has complementary with 3'UTR region of mRNA target: if the sequences pair perfectly, the mRNA is cleaved by the complex; instead, if the complementary is partial, there is only a reversible translational repression [Kappelmann M et al, 2013; Scott GK et al, 2007]. It is estimated that miRNA could regulate nearly 60% of all human protein-coding genes, each miRNA can modulate different mRNA, while a single gene can be targeted by multiple miRNAs [Zhang Y et al, 2011; Lebelo MT et al, 2019]. These findings suggest that miRNAs are involved in most cellular physiological processes, such as apoptosis proliferation, development, differentiation, metabolism [Cairns RA et al, 2011; Soga T, 2013; Romero-Cordoba SL et al, 2018; Thursby E et al, 2017; Carding S et al, 2015], but also in the onset of different pathology, especially tumorigenesis [Morrison DJ et al, 2016; Hong YH et al, 2005].

A lot of studies have investigated the role of miRNAs in cancerogenesis; analysis of miRNA expression profiles in tumor samples and normal tissues have displayed different patterns of overexpression or downregulation. miRNA dysregulation correlates with various human cancers and can function as either tumor suppressors by down-regulating oncogenic targets, or tumor promoters through negatively regulating tumor-suppressive target mRNAs. In this way miRNAs participate in the activation of cell proliferation or in the inactivation

of the apoptotic signaling pathway in conjunction with other genetic changes leading to cancer pathogenesis. A general finding is that global miRNA expression levels are lower in tumor tissues than normal tissues independent of cell type. In addition, poorly differentiated tumors present a lower global level of miRNA expression compared to more differentiated tumors. Several studies have investigated the action of miRNA-125. The different members of miR-125 family have been reported controversial properties in different types of cancer; they may contribute to the initiation and progression of cancers by acting as either tumor suppressors or oncogenes. miR-125 has been shown its tumor-suppressor functions in several cancers including ovarian cancer, bladder cancer and melanoma [Belkaid Y et al, 2013; Barbacid M et al, 1987; Fearon E et al, 2011]. In breast cancer, miR-125a and miR-125b were reported down-regulated in biopsy specimens and act as tumor suppressors by mediating the ERBB2 and ERBB3 pathway or by targeting the ETS1 gene [Elinay E et al, 2019; Georgieya K et al, 2015]. In addition, miRNAs do not only participate in proliferative and antiapoptotic processes in tumorigenesis but promote changing in cellular metabolism giving an adaptive advantage to cancer cells to fulfill the high energetic requirements for the maintenance of high proliferation rates, similarly, to grow at low oxygen concentrations and to use alternative carbon sources. These phenomena result from the dysregulated expression of different genes, including those encoding miRNAs [Sanders ME, 2000; Mcfarland LV et al, 2006; Rastmanesh R et al, 2011]. Obviously the large and important implication of these little molecules in cancer, drew attention to their possible use for pharmacological therapy. Pharmacological targeting of altered miRNAs may have therapeutic effects by suppressing relevant cancer signaling pathways without affecting normal cells.

1.6.1 microRNAs classification and databases

miRNAs are named with the prefix “miR”, followed by a dash and a number, which is based on the time when it was discovered. To miRNAs that diverge by a single or a couple of nucleotides, a letter is added to the end of the name (e.g. miR-124a is strictly correlated to miR-124b) in order to differentiate them.

Three letters can be added before miRNA's name, to indicate the species to which they belong (e.g: hsa-miR-124, where hsa stands for *H. sapiens*).
Datas about already discovered miRNAs are collected in international databases, of which the most updated and used is miRBase, which contains 2603 Human miRNAs. This database contains information on genetic regions, from which miRNAs are synthesized.

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Datas about already discovered miRNAs are collected in international databases, of which the most updated and used is miRBase, which contains 2603 Human miRNAs. This database contains information on genetic regions, from which miRNAs are synthesized.

1.6.2 Biogenesis and action mechanisms of microRNAs

miRNAs biogenesis starts from their precursors, which are mostly located in non-coding intergenic regions. The existence of a large amount of non-coding DNA in human genome made researchers consider the newly discovered DNA as "junk DNA", with no known functions. miRNAs' gene discovery inside genome, changed the original thoughts of scientific community [Lagos-Quintana M et al, 2006; Rodriguez A et al, 2004].

miRNAs biogenesis begins with RNA-polymerase II of pri-miRNAs, which are poly-adenylated and contain a cap at 5' end. Also the RNA polymerase III has a role in the synthesis of small RNAs which are involved in processes like cell growth and proliferation [Cramer P, 2004; Ohkuma Y, 2009; Woychik NA et al, 1990].

Once transcribed, pri-miRNAs present a sequence that causes the formation of a hairpin structure. This secondary structure represents a point of start in miRNAs maturation.

miRNA's maturation process begins inside the nucleus, where the microprocessor complex, composed by Drosha and Pasha proteins, reduces their dimension from 60 to 90 nucleotides, forming pre-miRNAs. These molecules are exported from nucleus to cytoplasm by Ran-5/exportin complex, which is GTP-dependent. In cytoplasm, a nuclease named Dicer processes once again pre-miRNAs, removing the loop structure and generating double-strand miRNAs, which are integrated with RNA inducing silencing complex (RISC), that also contains the Argonaut (Ago) protein, responsible for miRNA's inhibitory effects on mRNAs.

miRNA silencing process can be carried in two different ways: mRNA translation block or mRNA degradation. This depends on miRNAs-mRNAs base matching: if there is a perfect match between the sequences of miRNA and mRNA, the RISC complex cutting function is triggered and the mRNA is degraded; if there is only a partial mismatch between miRNA and mRNA, the first one binds 3'UTR region of mRNA, blocking the translation process [Lim LP et al, 2005].

1.6.3 Regulation of microRNAs expression

miRNAs expression regulation remains mostly unknown. Different tissues can have different levels of miRNAs expression. Endogenous and exogenous stimulation may regulate miRNAs expression, and also miRNAs may control other miRNAs expression [Stanczyk J et al, 2008; Lee RC et al, 1993].

Some studies show about 10% of miRNAs expression is controlled by DNA methylation [Lagos-Quintana M et al, 2003] and also hormones, dietary habits and hypoxia are involved in this regulation mechanism [Rodriguez A et al, 2004; Cramer P et al, 2004].

1.6.4 Pathogenetic roles of miRNAs

Despite the biological function of many miRNAs has not yet been characterized, the evaluation of their expression profile is important in order to obtain information about regulation mechanisms and physio-pathological implications. Many studies tested the implication of miRNAs in pathologies of different nature, from diabetes to neurological diseases, and have shown their validity as early-diagnosis markers and therapeutic follow-up markers.

Other studies demonstrated that miRNAs are also excellent biomarkers in cancer diagnosis, in fact their expression profile results altered in many tumors, suggesting their possible involvement in tumorigenesis [Mayr C et al, 2007; Hansen T et al, 2007].

Recently, miRNAs encoding genes have been classified as oncogenes and tumor suppressor genes [Wu W et al, 2007] depending on their function. It is not always clear if the alteration of miRNAs is directly correlated with cancer onset or if it is due to phenotypic variations of tumor cells. It is also important to note that a single miRNA could have both a role of oncogene and tumor suppressor, because of the variety of functions it performs inside the cell [Krek A et al, 2005].

Therefore, miRNAs involved in neoplastic processes are classified in two categories: oncomiRs and anti-oncomiRs, having respectively a promoting and a contrasting action towards tumorigenesis.

Different studies, carried out on mices, demonstrated how the inactivation of Dicer1, fundamental enzyme in miRNAs' processing, is positively correlated to

tumorigenesis [Lambertz I et al, 2010; Sekine S et al, 2009]. Also human DICER1 seems to have an important role as tumor suppressor (particularly on CRC cells), suggesting that miRNAs' biosynthesis is a key mechanism against tumorigenesis [Iliou MS et al, 2014].

1.6.5 Clinical role of miRNAs

miRNAs are mainly used to achieve additional diagnostic, prognostic and therapeutic parameters [Barbarotto E et al, 2008]. miRNA expression profile is very useful for a variety of purposes:

- Determine patient's prognostic parameters;
- To predict the potential effectiveness of a particular therapy;
- To study about ethnicity susceptibility to cancer and metastasis;
- To investigate about single patient's susceptibilities.

miRNA expression profiling is a valid instrument in lung cancer, because it permits to distinguish between normal lung cells and cancer cells and it gives useful prognostic information [Yanaihara N et al, 2006]. miRNA expression profiling is also used to differentiate normal cells from tumor cells in Chronic lymphocytic leukemia. It could be also used to trace back from undifferentiated tumor to the primary tumor from which it derives [Calin GA et al, 2005].

1.6.6 miRNAs detection methods

In the past, miRNAs were identified using glass slide microarray techniques, which consent to evaluate the expression profile of multiple miRNAs in a single sample [Babak T et al, 2004; Nelson PT et al, 2004; Barad O et al, 2004; Sole Y et al, 2004]. This method is based on the hybridization between thousands of oligonucleotides, covalently bound to a glass support, and specifically marked miRNAs. In addition to this traditional technique, it is possible to use other methods like the quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), which is also capable of identifying different miRNA's transcription patterns, highlighting those directly involved in certain pathologies. Recent studies evaluated the presence of miRNAs in body fluids like saliva, serum and urine [Crimi S et al, 2020].

The origin of circulating miRNAs is not yet clear, but they are supposed to circulate inside exosomes which could derive from normal or tumoral cells [Wang K et al, 2010]. Exosomes both protect miRNAs from degradation and stabilize them even in critical experimental conditions [Chen X et al, 2008; Mitchell PS et al, 2008]. Other hypotheses suggest that circulating miRNAs derive from lysed cells and their stability is due to their bond with DNA, lipids and proteins [Chen X et al, 2008; El-Hefnawy et al, 2004; Sisco KL et al, 2001].
The use of miRNAs as potential biomarkers in body fluids is still a controversial topic and needs further studies to evaluate the normal levels of expression and the presence of different miRNAs in relation with various factors that could affect their expression profile, as:

- Different body fluids;
- Cancer therapy, including chemotherapy, surgery, radiation or their combination;
- Ethnicity, gender and age.

Other critical issues of circulating miRNAs usage as prognostic factors are the lack of understanding of their releasing mechanism in body fluids and the absence of an unequivocal technological approach to validate their expression patterns. The variability in manipulation techniques and investigation methods could affect the real expression profile.

1.6.7 Other applications of miRNAs

A new application field of miRNAs, which makes them predictor of patients' response to a specific therapy, is in development. Different chemotherapy agents could determine several responses depending on miRNAs expression patterns [Lin PL et al, 2014; Wang P et al, 2015]
An interesting possibility is represented by the use of miRNAs as targeted therapy, by the usage of *antagomir*, chemically modified oligonucleotides that bounds competitively miRNAs, inhibiting their functions [Krutzfeldt J et al, 2005].
One application of this therapy is conducted in liver metabolic diseases, targeting miR-122. An in vivo study carried on both obese and normal mouses, targeting

miR-122 in the liver. Both of them displayed a decrease in plasma cholesterol [Esau C et al, 2006]. Stability, specificity and a low cytotoxicity make antagomir a novel therapy for disease treatment.

Antagomir usage as target therapy shows some limitations:

- A better comprehension of their action mechanism is needed, as well as their characterization and categorization, despite the great step forward made by computational bioinformatic, that is not enough anyway to identify with accuracy all genetic targets and functions of miRNAs inside the cell [Zhang B et al, 2006]
- It is not simple to formulate a drug based on this technology, due to miRNA stability. An attempt was made by using viral vectors as vehicles of antagomir, with poor results in humans due to their capability to generate auto-immune response [Lefesvre P et al, 2002; Rubinson DA et al, 2003; Szulc J et al, 2006; Thomas CE et al, 2004; Yang Y et al, 1995]

1.7 microRNAs in Colorectal Cancer

Various in vitro studies identified mRNAs involved in the most common altered pathways involved in colorectal cancer development.

A valid alternative to mRNA profiling is represented by the study of miRNAs expression patterns in CRC patients, which permit to have a much more consistent point of view by confronting miRNAs' expression level between cases and controls or between patients' pathologic and normal bioptic samples. miRNA expression levels are evaluated by using microarray platforms, real-time PCR and, more recently, digital droplets PCR methods (ddPCR).

1.7.1 miRNAs interaction with known CRC-related molecular drivers

Wnt signaling pathway is the most altered in CRC, with mutations in APC/β-catenin genes (found in over 75% of CRC cases). miRNAs could interfere with this signaling pathway, having a regulatory role in tumorigenesis. This suggests that Wnt-modulatory miRNAs could represent a possible target for future therapies, considering Wnt central role in CRC.

Among Wnt-interacting miRNAs, miR-135a and miR-135b represent well-known examples. They are over-expressed in CRC and down-regulate APC levels, leading to an increase in Wnt activation level [Nagel R et al, 2008].

The miRNA family of miR-34 (mir-34a/b/c) represents an example of antioncomiR, by inhibiting different effectors of Wnt pathway, like WNT1, WNT3, LRP6, β-catenin and LEF1 [Kim NH et al, 2011]. TP53 triggers miR-34's transcription, explaining how TP53 itself may inhibit Wnt pathway.

In more than half of all CRC cases, a GOF mutation was observed in KRAS and BRAF genes, which activate MAPK pathway, stimulating cell proliferation processes [Cancer Genome Atlas Network, 2012; Guinney J et al, 2015].

In CRC, miR-31 seems to be a powerful KRAS enhancer, by negatively regulating RASA1, a KRAS' inhibitor [Kent OA et al, 2016].

BRAF mutations are frequently found in proximal colon tumors, prevalently correlated to microsatellite instability (MSI) [Domingo E et al, 2004]. There are no scientific evidence of a correlation between miRNAs and BRAF, although BRAF seems to be a target for miR-378, an anti-oncomiR in CRC [Wang Z et al, 2015]. miR-31 also could be associated twithBRAF mutations and to a more aggressive cancer phenotype [Nosho K et al, 2014; Ito M et al, 2014; Choi YW et al, 2016].

2. AIM OF THE STUDY

Despite the screening programs available for the early identification of colorectal cancer, a significant fraction of patients is diagnosed late when the tumor is in an advanced stage with a consequent worse prognosis for the patients. Indeed, at present there are no effective humoral biomarkers for the early diagnosis of this pathology. Therefore, there is an urgent need to discover novel reliable diagnostic and prognostic factors for colorectal cancer.

As described in the previous paragraphs, several studies have tried to identify novel effective biomarkers for the diagnosis of colorectal cancer, however, no concordant results were obtained on this matter. In this context, several studies have identified some microRNAs (miRNAs) as potential biomarkers for different pathologies and tumors. miRNAs represent potential effective biomarkers as they can be released by cancer cells in body fluids; therefore, some studies have proposed the evaluation of circulating miRNAs to predict the risk of development of different tumors. However, no verified results have been obtained for colorectal cancer.

On these bases, the aim of the present study was to computationally identify a set of miRNAs associated with the development and progression of colorectal cancer in order to validate their diagnostic and prognostic potential in a pilot cohort of CRC patients and healthy controls. In particular, first a bioinformatics analysis was performed by analyzing the computational data contained in The Cancer Genome Atlas (TCGA) and GEO DataSets databases in order to identify a list of miRNAs associated with the development of colorectal cancer. Further bioinformatics prediction tools were used to establish the functional role of these miRNAs in colorectal cancer. Subsequently, the *in silico* data were validated in both FFPE and liquid biopsy samples obtained from colorectal cancer patients and healthy controls by using the high-sensitive droplet digital PCR (ddPCR) amplification systems. After ddPCR analyses, the diagnostic potential of four selected miRNAs, hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-503-5p and hsa-miR-375 was assessed through statistical analyses and further bioinformatics evaluations.

3. MATERIALS AND METHODS

3.1 Computational Identification of microRNAs Involved in the Development and Progression of Colorectal Cancer

The computational evaluation of miRNA expression bioinformatics data contained in the Gene Expression Omnibus DataSets (GEO DataSets) and referred to colorectal cancer samples was performed by using different computational approaches.

First, the miRNA expression microarray datasets were selected by performing an advanced search in the GEO DataSets database. For this analysis, the following search terms were used: "(("non-coding RNA profiling by array"[DataSet Type]) AND colorectal cancer) AND "Homo sapiens"[porgn:__txid9606]".

These search criteria allowed us to identify a total of 113 datasets (until March 2019), however, only those datasets fitting with further exclusion and inclusion criteria were selected to strengthen our analysis.

The following selection criteria were used:

Inclusion criteria

- Datasets containing at least 30 samples (both normal and tumor samples);
- Datasets containing miRNA expression data of CRC patients and healthy controls;

Exclusion criteria

- Datasets containing only normal or only tumor samples;
- Datasets containing miRNA expression data of animal models or human cell lines;
- Datasets with annotation information not available

By applying all these criteria it was possible to select ten different datasets. Table 1 contains the datasets selected for the subsequent computational analyses (Table 1).

Table 1. Main features of the selected datasets.

Series Accession	n. normal	n. cancer	Samples	Platform	Author ref.	Total Samples
GSE18392	29	116	Normal colon tissues and colon tumor tissues	Illumina Human v1 MicroRNA expression beadchip	Sarver AL et al, 2009. BMC Cancer. 9: 401.	145
GSE108153	21	21	Paired tumour tissues and adjacent normal tissues	Agilent-046064 Unrestricted Human miRNA V19.0 Microarray	Zeng Z et al, 2017 (NO REF)	42
GSE30454	20	54	Normal colonic mucosa and RNA from formalin-fixed paraffin-embedded tissue blocks from 4 different CRC groups	Illumina Human v2 MicroRNA expression beadchip	Balaguer F et al, 2011. Clin Cancer Res. 17: 6239-49.	74
GSE35834	23	31	Normal adjacent mucosa, primitive colorectal cancer and liver metastasis tissues	[miRNA-1_0] Affymetrix miRNA Array	Pizzini S et al, 2013. BMC Genomics. 14: 589.	78
GSE38389	71	69	Tumor biopsies and corresponding matched mucosa samples	Exiqon miRCURY LNA microRNA array v.9.2 Extended Version	Gaedcke J et al, 2012. Clin Cancer Res. 18: 4919-30.	140
GSE41012	15	20	CR distant normal mucosa and different stages of CR primary tumor	Exiqon miRCURY LNA microRNA Array, v. 9.2, all organisms	Li X, 2015. (NO REF)	35
GSE41655	15	33	Human colorectal tissues, including normal mucosa, adenoma and adenocarcinoma.	Agilent-021827 Human miRNA Microarray [miRNA_107_Sep09_2_105]	Shi X, Zhang Y, Cao B, et al, 2015 (NO REF)	107
GSE49246	40	40	Adjacent normal tissues and stage II colon tumor tissues	Sun Yat-Sen University Cancer Center Human microRNA array	Zhang JX et al, 2013. Lancet Oncol. 14: 1295-306.	80
GSE68204	8	37	Normal rectal biopsies and tumor rectal biopsies	Agilent-021827 Human miRNA Microarray (V3) (miRBase release 12.0 miRNA ID version)	Millino C et al, 2017. J Cell Physiol. 232: 426-435.	125
GSE83924	20	20	Fresh frozen tissue samples from tubular and tubulovillous adenoma and colorectal adenocarcinoma	[miRNA-3] Affymetrix Multispecies miRNA-3 Array	Nagy ZB, Wichmann B, Molnár B, 2016. (NO REF)	60

For all the selected datasets, the differential analyses between the expression levels of miRNAs observed in colorectal cancer samples compared to those observed in were performed. More in detail, differential analyses were performed using the GEO2R tool already available within the GEO DataSets database. This software, automatically normalizes the expression data of miRNAs observed in different samples and perform differential analyses between two group of samples. In this study differential analyses were performed among “CRC samples” vs “Normal samples”. The values of differential expression were expressed as base 2 logarithm of the fold change (log2FC). Only differentially expressed miRNAs with a p-value $p < 0.01$ were considered as potentially involved in colorectal cancer development and progression to strengthen our results.

Thus, the data matrix of each dataset was downloaded and analyzed through GEO2R and the miRNAs significantly dysregulated ($p<0.01$) were annotated using the latest nomenclature available published by miRBase (miRBase V 22) (http://www.mirbase.org/).

The lists of dysregulated miRNAs obtained from each datasets were merged together using the Venn Diagrams tool of the Bioinformatics & Evolutionary Genomics (BEG) (http://bioinformatics.psb.ugent.be/webtools/Venn/). This approach allowed us to identify only the miRNAs strongly up-regulated or down-regulated in at least 3 of the previously selected datasets.

From these merged miRNAs, a further selection was performed to select only miRNAs with a strong involvement in CRC. Therefore, Top 20 list of dysregulated miRNAs was obtained for merged miRNAs. In particular, the 10 most up-regulated and down-regulated miRNAs in colorectal cancer patients compared to healthy controls.

This approach allowed us to identify only the miRNAs strongly up-regulated or down-regulated in at least 3 of the previously selected datasets. For each miRNA the dysregulation levels (over-expressed or down-regulated) were displayed using red boxes and blue boxes for up- and down-regulation, respectively. Red and blue boxes were of different gradients of darkness according to the expression levels observed. In particular, miRNAs were divided into "highly" ($logFC \geq 3$), "moderately" ($logFC\ 1.5 < x < 3$), "lightly" ($logFC\ 0.5 < x < 1.5$) and "poorly" ($logFC\ 0 < x < 0.5$) up-regulated or down-regulated (negative logFC values).

3.2 Association between the Computationally Selected miRNAs and the Main Genes Altered in Colorectal Cancer

In order to correlate the miRNAs computationally identified with the main genes involved in the development and progression of colorectal cancer, the Catalogue of Somatic Mutation in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic) was used to select the ten most mutated and altered genes in colorectal cancer. After the identification of the ten most altered genes in CRC, the interaction levels between these genes and the selected miRNAs were established by using the bioinformatics prediction tool microRNA Data Integration Portal (mirDIP – V 4.1.1.6, Nov 2017) (http://ophid.utoronto.ca/mirDIP). In particular, mirDIP, this software is able to integrate the bioinformatics prediction data contained in 26 different databases of miRNAs-mRNA interaction. The levels of interaction between the miRNAs and the targeted gene are expressed as very high, high, medium and low according to the integrated score calculated by the mirDIP algorithm.

3.3 Molecular Pathways Modulated by the Selected miRNAs and their Functional Role

To further evaluate the functional role of the computationally identified miRNAs, further bioinformatics approaches were used. In particular, the bioinformatics prediction tool DIANA-mirPath (v.3) was used to perform a pathway prediction analysis aimed at investigating the role of these miRNAs in the modulation of the cellular and molecular pathways mostly involved in the development of tumors. Besides the molecular pathway modulated by the selected miRNAs, through the DIANA-mirPath analysis it was also possible to identify a list of targeted genes.

More in detail, two different analyses were performed through DIANA-mirPath. In the first analysis, the most altered pathways altered in CRC according to literature were analyzed. In particular, specific pathways for colon cancer were used as search terms and analyzed, following the indications given by The Cancer Genome Atlas Network in reference to CRC.

In the second analysis, the computationally identified miRNAs were used as search terms in order to identify the genes and pathways altered by them. Cancer pathways of other tumor types and the molecular pathways not directly involved in cancer development or in cell cycle and homeostasis, such as Hepatitis B pathway (hsa05161), Axon guidance pathway (hsa04360), Lysine degradation pathway (hsa00310), etc., were excluded from this analysis.

3.4 Patients and Sample Included in the Study

To validate the computational results obtained through the analysis of miRNA expression data as well as to confirm the diagnostic or prognostic value of the computationally selected miRNAs, a case series of liquid biopsy and Formalin-Fixed Paraffin-Embedded (FFPE) samples obtained from colorectal cancer patients and healthy controls were analyzed. As regards the analysis performed on FFPE samples, a cohort of 20 patients with a confirmed diagnosis of CRC was enrolled. In particular, for each CRC patient, cancer tissues and normal adjacent mucosa were collected. Ten FFPE sections of 5-8 μm were obtained from each FFPE sample (tumor or adjacent normal mucosa).

In addition, liquid biopsy samples obtained from a pilot cohort of 15 CRC patients and 15 healthy controls were collected to evaluate the expression levels of the four computationally selected miRNAs. Briefly, from both patients and controls, two peripheral blood draws were collected in order to separate serum (tube with separating gel) and plasma, buffy coat and red cells (tube with K3 EDTA) for future analysis. To obtain the different sample types, blood samples were centrifuged at 2,000 g for 10 minutes at room temperature in order to obtain different aliquots of serum, plasma, buffy coat and red cells. The samples were then stored at -80°C until their use.

3.5 RNA Extraction and Reverse Transcription of microRNA

For the extraction of miRNAs from FFPE tumors and adjacent normal tissues, the following protocol was adopted. Total RNA, including miRNAs, was extracted by using the miRNeasy FFPE kit (Qiagen – Cat. N. 217504) and 2-3 tissue sections of 5-8 μm following the manufacturer's instruction.

As regards the liquid biopsy samples, as previously mentioned, miRNAs can be easily detected in different biological fluids representing optimal non-invasive biomarkers for the diagnosis of tumors and other pathologies diseases. By using the protocol described below, the circulating miRNAs were extracted from both serum samples obtained as described in the previous chapter.

For the extraction of circulating miRNAs, the miRNeasy Serum/Plasma kit (Cat. No. 217184, Qiagen, Hilden, Germany) was used modifying some steps of the analysis.

Briefly, serum samples were centrifuged at 2,000 g × 10 min at room temperature to pellet down debris and protein aggregates. Then, 200 μL of samples was used in the following protocol. Before the extraction, the exogenous synthetic UniSP4 (Cat. No 219610, Qiagen, Hilden, Germany) was added to the samples to normalize the absolute quantification of miRNA expression levels and evaluate extraction efficiency. Molecular grade reagents (ethanol and chloroform) were used during the extraction procedure.

After RNA extraction, 2 μL of total RNA, including miRNAs, was reverse transcribed into cDNA using the miRCURY LNA RT kit (Qiagen - Cat. No. 339340).

The cDNA obtained from the FFPE tissues was firstly diluted 1:50 in RNase-free water while the cDNA obtained from the liquid biopsy samples was analyzed directly.

3.6 ddPCR miRNA Quantification

For the analysis of miRNA expression levels in both FFPE and liquid biopsy samples, a custom Qiagen-Bio-Rad hybrid protocol was adopted using specific primers specific for the four selected miRNAs as well as for the exogenous normalizer Unisp4 spike-in control (miRCURY LNA miRNA PCR Assays x200, Qiagen - Cat. No. 339306). For the analysis of miRNA expression levels from FFPE tissues, the expression levels of miRNAs were normalized according to the endogenous control U6 snRNA.

In particular, ddPCR was used to better evaluate the expression levels of miRNAs in low-quality samples as FFPE tissues or in samples with a low amount of miRNAs as liquid biopsy samples.

ddPCR is used in a similar way to classical PCR, with the difference that in ddPCR the sample is nano-partitioned in thousands of droplets were single reactions were run. In particular, the DNA target and reaction reagents are partitioned in water-oil droplets in the ddPCR technology. Thus, theoretically, each droplet contains specific primers and probes, Taq polymerase and the amplification buffer and the target of investigation. The water-oil droplet is generated with a droplet generator and the droplets are subsequently amplified through a classic PCR amplification protocol. After amplification, ~20.000 droplets are read using a droplet reader to evaluate the fluorescent signals of each droplet a CCD camera.

In particular, the ddPCR reaction mix was prepared as follow:

- 11 µL of 2x QX200 ddPCR EvaGreen® Supermix (Cat. No. 1864034 - Bio-Rad, Hercules, California, USA);
- 1.1 µL of miRNA-specific target probe (Thermo Fisher Scientific - A25576);
- 6.9 µL of free-water RNase and DNase;
- 3 µL of cDNA to obtain a final volume of 22 µL.

After the preparation of the reaction mix, 20 µL of samples were used to generate about 20,000 droplets with the QX200 droplet generator (Bio-Rad, Hercules, California, USA). After generation, droplets were transferred to a 96-well plate,

sealed and amplified in a C1000 Thermal Cycler (Bio-Rad, Hercules, California, USA) using the following thermal conditions:

- polymerase activation for 10 minutes at 95°C,
- 40 cycles of amplification at 94°C for 30 seconds (denaturation) and 60°C for 1 minute (annealing)
- droplet stabilization at 98°C for 10 minutes
- Infinite hold at 4°C.

A ramp rate of 2°C/s was used among all the amplification steps. After amplification, negative and positive droplets were read in the QX200 Droplet Reader (Bio-Rad, Hercules, California, USA). All experiments were performed in triplicate (Figure 6).

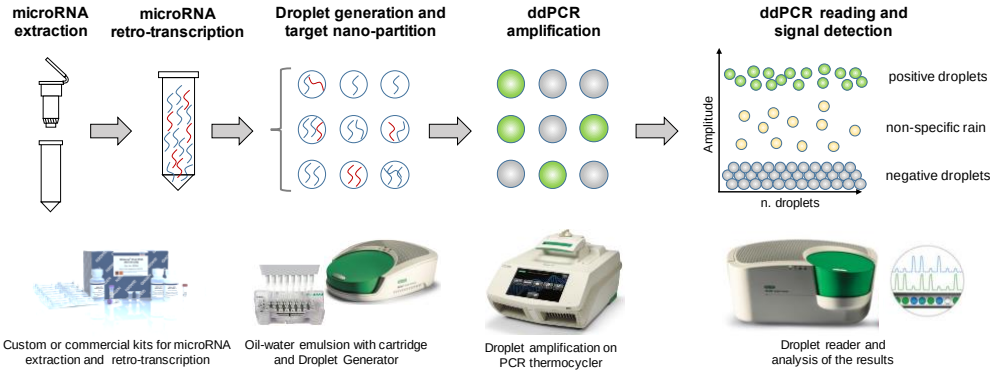

Figure 6. ddPCR workflow for the analysis of miRNAs.

3.7 Bioinformatics Analyses

After the validation of the diagnostic potential of the four selected miRNAs, hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-503-5p and hsa-miR-375, further bioinformatics analyses were performed in order to evaluate the correlation of these miRNAs with clinical-pathological features of CRC patients.

First, the miRTargetLink Human bioinformatics software (https://ccb-web.cs.uni-saarland.de/mirtargetlink/index.php) was used to identify the genes targeted by the four miRNAs investigated through ddPCR. Through this software it was possible to identify the genes targeted by the four miRNAs through the analysis of the miRNA-mRNA interaction data contained in different miRNA-target databases, including KEGG Pathway, miRBase,

TargetScan, etc. In particular, two analyses were performed, one for the identification of genes strongly targeted by the selected miRNAs and the second one for the identification of genes with weak interaction with the selected miRNAs.

The genes strongly modulated by the four selected miRNAs and identified through the miRTargetLink Human analysis were further analyzed with GO enrichment tools like the Search Tool Retrieval of Interacting Genes/Proteins (STRING) and GO Panther software to establish the functional roles of the miRNA-targeted genes in CRC.

Finally, clinical data contained in the TCGA COAD and TCGA READ databases were analyzed by using two different tools. In particular, Gene Expression Profiling Interactive Analysis (GEPIA) was used to evaluate the dysregulation of the genes targeted by the four validated miRNAs (http://gepia.cancer-pku.cn), while OncoLnc was used to evaluate the prognostic significance of the selected miRNAs in predicting the overall survival of COAD and READ patients (http://www.oncolnc.org).

3.8 Statistical Analyses

As regards the computational analyses performed to identify a set of miRNAs potentially involved in the development and progression of colorectal cancer, all the expression data of miRNAs in the selected GSE datasets were already normalized by GEO2R software, therefore, no additional normalization procedures were applied to data obtained from all datasets included in this study. The statistical significance of the differentially expressed miRNAs was also calculated by GEO2R. Similarly, the statistical analyses related to the targeted genes and pathways were automatically performed by mirDIP and DIANA-miPath software.

As regards the molecular data obtained through ddPCR, the miRNA expression levels observed in liquid biopsy samples were normalized according to the expression levels of the exogenous control UniSp4, while the miRNA expression levels observed in FFPE samples were normalized according to the expression levels of the endogenous control RNA U6.

The ddPCR raw data were analyzed through the QuantaSoft software (Bio-Rad, Hercules, California, USA) for absolute quantification of miRNA expression in serum and FFPE tissue samples. As regards the statistical analyses performed on these data, the Kolmogorov-Smirnov normality test was used to evaluate the distribution of expression levels of hsa-miR-21-5p, hsa-miR-497-5p, hsa-miR-503-5p and hsa-miR-375. Wilcoxon test was used to establish the statistical differences existing between tumor FFPE samples and adjacent normal mucosa. For the statistical analysis of miRNA expression levels in liquid biopsy samples the Mann-Whitney test was used. To assess the specificity and sensitivity of the miRNAs analyzed, Receiver Operating Characteristic (ROC) curves were calculated. All statistical analyses were performed using GraphPad Prism v.8.

4. RESULTS

4.1 Identification of miRNAs Involved in Colorectal Cancer Development and their Functional Role

As previously mentioned, the search for colorectal cancer miRNA expression datasets resulted in the identification of a total of 113 miRNA expression datasets. Of these, only ten met the inclusion and exclusion criteria adopted.

The differential analyses performed on the expression levels of miRNAs between colorectal cancer samples and normal controls allowed the identification of 39 dysregulated miRNAs in colorectal cancer patients compared to control. Among these miRNAs, only those with concordant expression levels among the datasets where they were expressed were selected. In addition, by considering only the TOP 20 dysregulated miRNAs, a list of 20 strongly altered miRNAs in colorectal cancer was obtained (Figure 7).

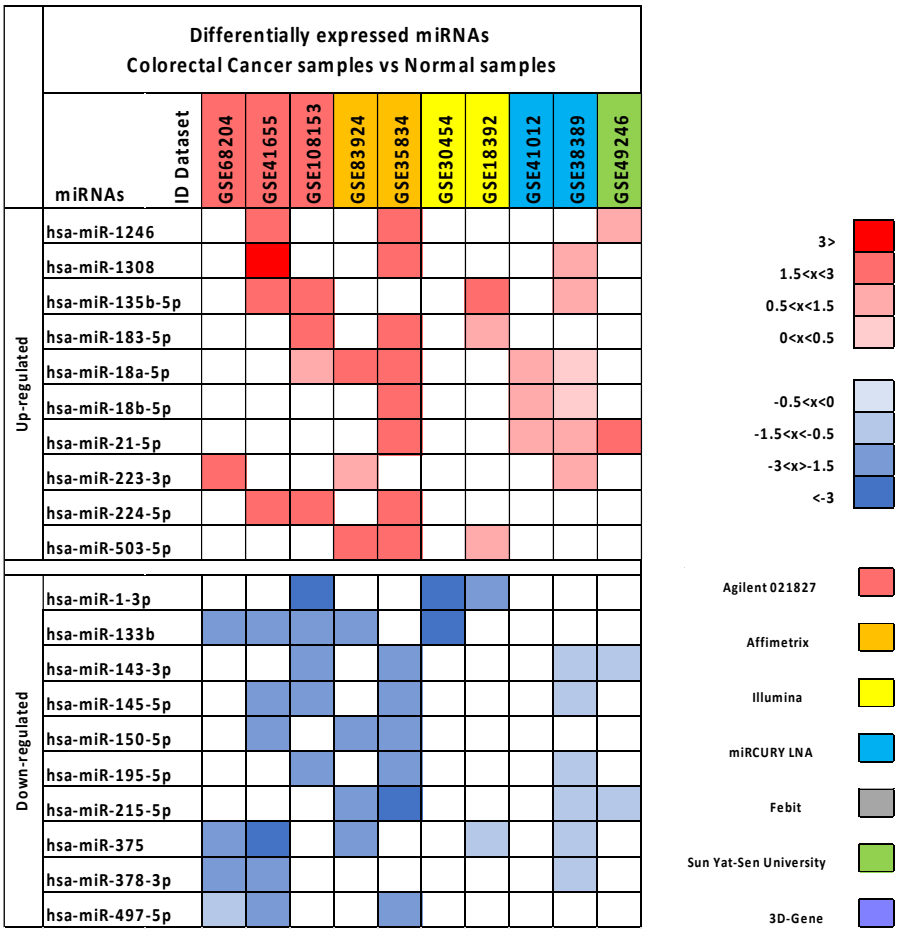

Figure 7. Top 20 dysregulated miRNAs in colorectal cancer.

In particular, ten miRNAs were up-regulated, hsa-miR-1246, hsa-miR-1308, hsa-miR-135b-5p, hsa-miR-183-5p, hsa-miR-18a-5p, hsa-miR-18b-5p, hsa-miR-21-5p, hsa-miR-223-3p, hsa-miR-224-5p and hsa-miR-503-5p and ten miRNAs were down-regulated, i.e. hsa-miR-1-3p, hsa-miR-133b, hsa-miR-143-3p, hsa-miR-145-5p, hsa-miR-150-5p, hsa-miR-195, hsa-miR-215-5p, hsa-miR-375, hsa-miR-378-3p and hsa-miR-497-5p (Figure 7).

Some of these miRNAs are known to be involved in the alteration of different signal transduction pathways responsible for the development of tumors acting as oncomiR or antioncomiR thus resulting up-regulated and down-regulated, respectively.

Among these 20 miRNAs, some have been already associated with the development of tumors, including colorectal cancer. In particular, hsa-miR-21-5p and hsa-miR-375 were, respectively, up-regulated and down-regulated in five out of ten datasets analyzed. Supporting these data, some studies have already described the involvement of these miRNAs in the development of colorectal cancer [Cui F et al, 2016; Zaharie F et al, 2015]. In particular, hsa-miR-375 acts as an antioncomiR blocking the proliferation of cancer cells and the formation of metastases. Therefore, the down-regulation of this miRNA in colorectal cancer supports its involvement in tumor development. Contrarily, hsa-miR-21-5p acts as an oncomiR resulting up-regulated in colorectal cancer and in tumors in general [Sazanov AA et al, 2016].

Through this analysis, other miRNAs have emerged: the most up-regulated miRNAs were hsa-miR-1246, hsa-miR-18a-5p, hsamiR-223-3p; while the most down-regulated miRNAs were hsa-miR-133b, hsa-miR-215-5p.

Of note, hsa-miR-1308 was not considered for the further computational analyses because it is a fragment of tRNA and not a real miRNA.

To better elucidate the functional roles of these twenty dysregulated miRNAs, further bioinformatics analyses were performed. First, the ten most altered genes in colorectal cancer were identified by consulting the data contained in the COSMIC database. Through these analyses, the following genes were identified:

APC (51%), TP53 (46%), KRAS (34%), FAT4 (23%), LRP1B (20%), TGFBR2 (17%), PIK3CA (15%), KMT2C (13%), ZFHX3 (12%) e BRAF (10%) (Figure 8).

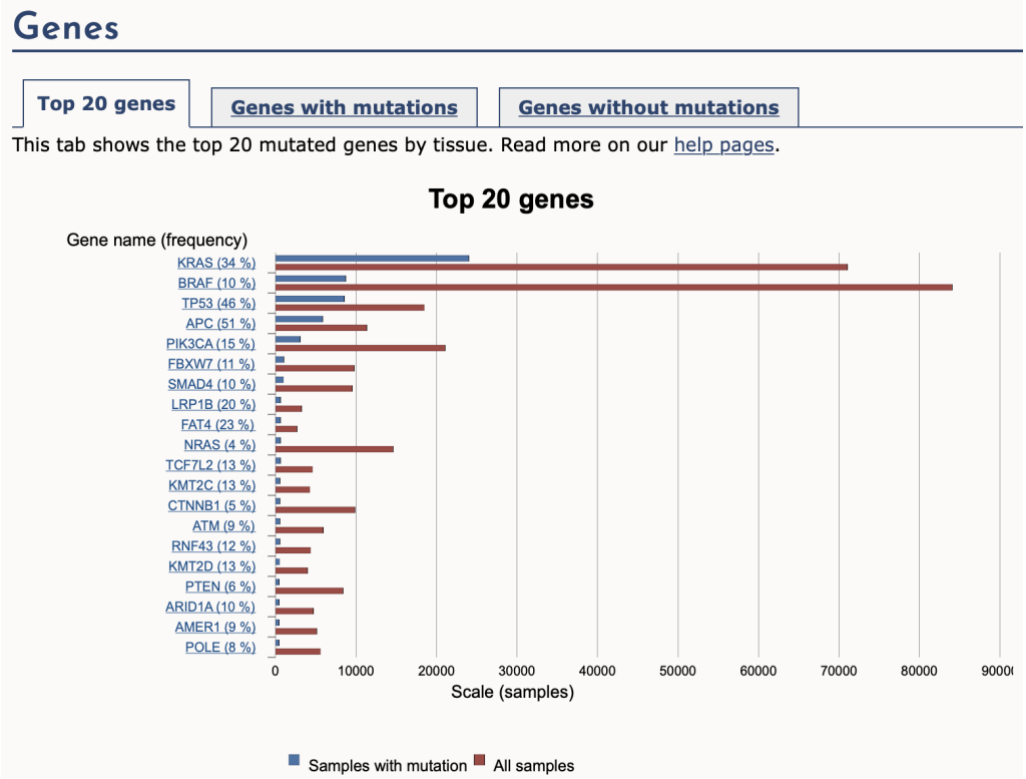

Figure 8. Main mutated and altered genes in colorectal cancer

To establish the interaction existing between the 19 selected miRNAs and the top ten altered genes in colorectal cancer, the mirDIP analysis was performed. Through this analysis it was possible to observe medium-high levels of interaction between the selected miRNAs and the altered genes. Among genes, ZFHX3 and KMT2C were the most altered by the selected miRNAs. While, taking into account the up-regulated miRNAs, those with the higher interaction levels with the selected altered genes were hsa-miR-223-3p, hsa-miR-183-5p; among the down-regulated miRNAs those with the highest interaction levels were hsa-miR-195-5p, hsa-miR-150-5p and hsa-miR-375 (Figure 9).

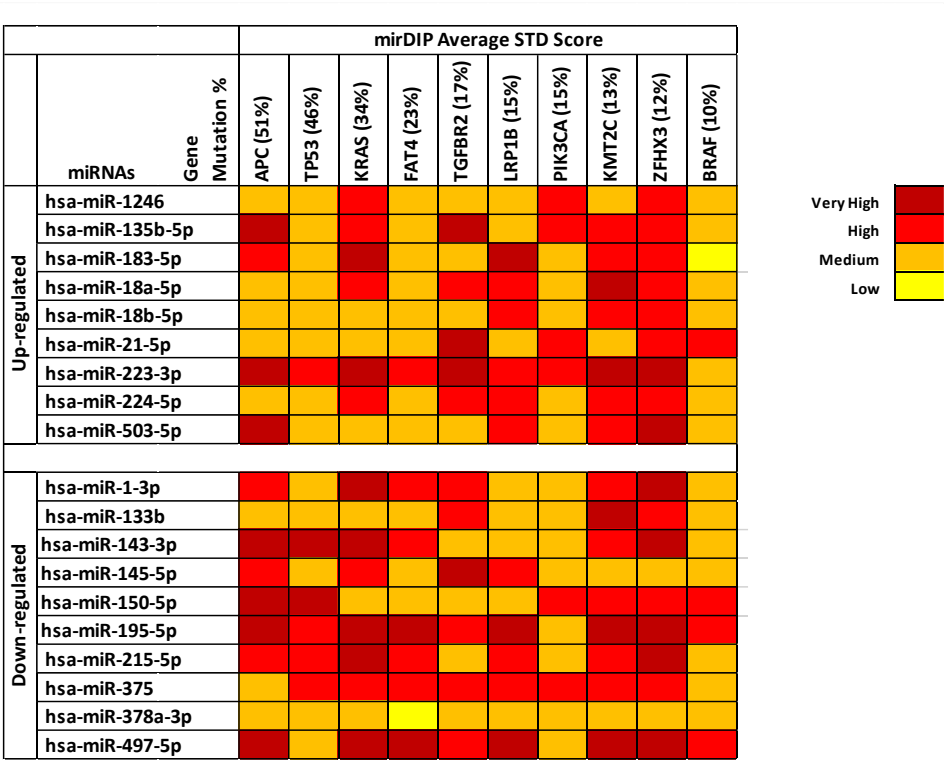

Figure 9. miRNA-mRNA interaction levels between the computationally selected miRNAs and main altered genes in CRC.

To further evaluate the functional role of miRNAs in cancer development, the DIANA-mirPath analysis of the 19 selected highly-modulated miRNAs was performed. As described in the methods section, two different approaches were performed. In the first approach the pathway prediction analysis was performed searching the most altered pathways in CRC.

As shown in Figure 10, the seven most altered pathways according to the TCGA data were analyzed. The analysis revealed as all the selected miRNAs were able to modulate the Wnt signaling pathway (hsa04310), RAS-MAPK signaling pathways (hsa04041 and hsa04010 respectively), PI3K-AKT signaling pathway (hsa04151), TGF-β and p53 signaling pathways (hsa04350 and hsa04115 respectively) and the mismatch repair pathway (hsa03430). The most modulated pathways were the MAPK signaling pathways (hsa04010) and the PI3K-AKT signaling pathway (hsa04151). Among the most involved miRNAs, the up-regulated hsa-miR-183-5p and hsa-miR-21-5p and the down-regulated miRNAs hsa-miR-195-5p and hsa-miR-497-5p were those able to target the higher number of genes (a total of 214 genes) (Figure 10). These miRNAs were thus

able to target key genes involved in these pathways and in cancer development such as TP53, APC, several proteins of the WNT family (WNT3A, WNT5A and WNT9A) and of the MAPK family (MAPK1, MAPK8 and MAPK9), VEGFA and MYC suggesting their possible use in diagnostic and clinical practice (Figure 10).

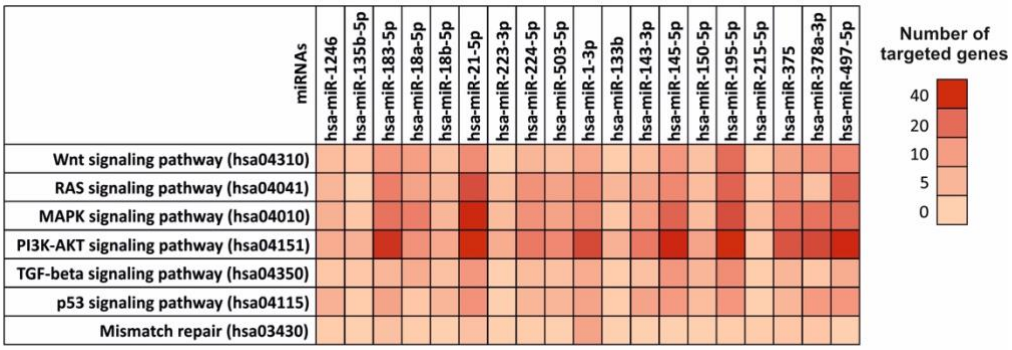

Figure 10. Interaction between the selected miRNAs and TCGA colorectal cancer pathways according to DIANA-mirPath v.3

Taking into account the 19 miRNAs identified, the altered genes and pathways were identified through the DIANA-mirPath second approach.

This second analysis revealed a total of 15 different cancer-related molecular pathways altered by the selected miRNAs of which three were shared with the previous analysis (PI3K-AKT signaling pathway, TGF-β and p53 signaling pathways (hsa04350 and hsa04115).

This analysis revealed that the selected miRNAs were able to modulate a total of 460 univocal genes belonging to different molecular pathways. In Figure11, the most altered pathways involved in tumor progression are shown. summarized the predicted pathways involved in cancer development and targeted by the 19 computationally selected miRNAs and their interaction with all genes of these pathways (Figure 11). All miRNAs showed to modulate the molecular pathways involved in cancer development, excluding the miRNAs hsa-miR-503-5p, hsa-miR-1-3p and hsa-miR-215-5p that have not shown interactions with any pathways. In addition, this approach confirmed the weak interaction of hsa-miR-215-5p, hsa-miR-135b-5p, hsa-miR-223-3p and hsa-miR-133b with the molecular pathways taken into account (Figure 11).

The miRNAs able to target the highest number of genes in these 15 pathways were hsa-miR-145-5p, hsa-miR-195-5p, hsa-miR-378a-3p and hsa-miR-497-5p.

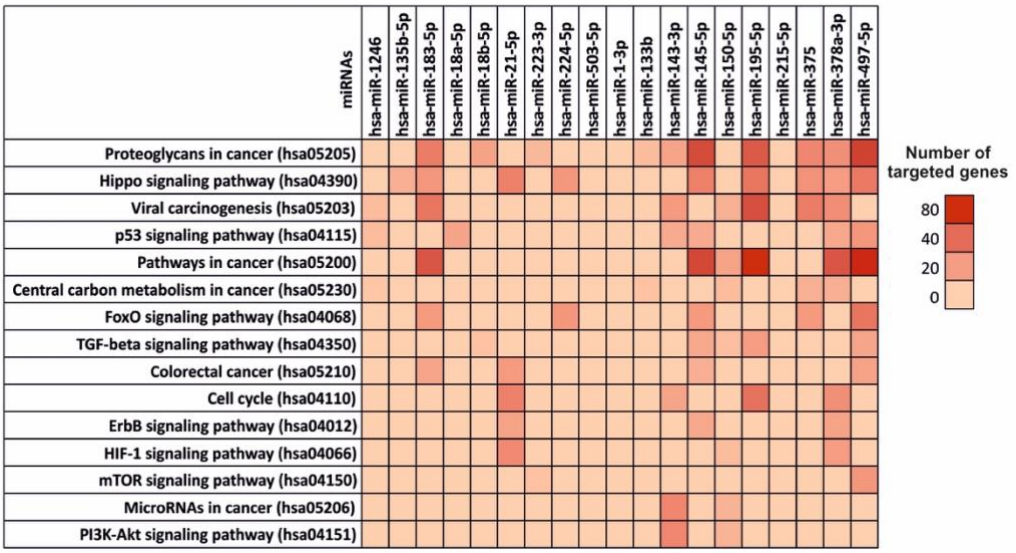

Figure 11. Interaction between selected miRNAs and several molecular pathways involved in cancer development according to DIANA-mirPath analysis.

Overall, these two approaches confirmed that the selected miRNAs were strongly involved in the modulation of genes and pathways involved in the development of tumors, including colorectal cancer.

Therefore, such miRNAs have not only a role as diagnostic markers of CRC, but could also mediate directly the processes that lead to the development colorectal cancer.

From these computational analyses, four miRNAs were selected for the validation analyses performed in FFPE and liquid biopsy samples. In particular, the two up-regulated miRNAs, hsa-miR-21-5p and hsa-miR-503-5p, and two down-regulated miRNAs, hsa-miR-375 and hsa-miR-497-5p, were selected for the ddPCR analyses.

4.2 Validation of the Expression Levels of the Four Selected miRNAs in Colorectal Cancer Samples

In order to validate the potential diagnostic value of hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p, the expression levels of these miRNAs were first evaluated in FFPE tissue samples obtained from colorectal cancer patients. In particular, for each patient both tumor and normal adjacent FFPE tissues were collected.

For all the miRNAs tested and the endogenous control RNA U6 good amplification signals were obtained (Figure 12).

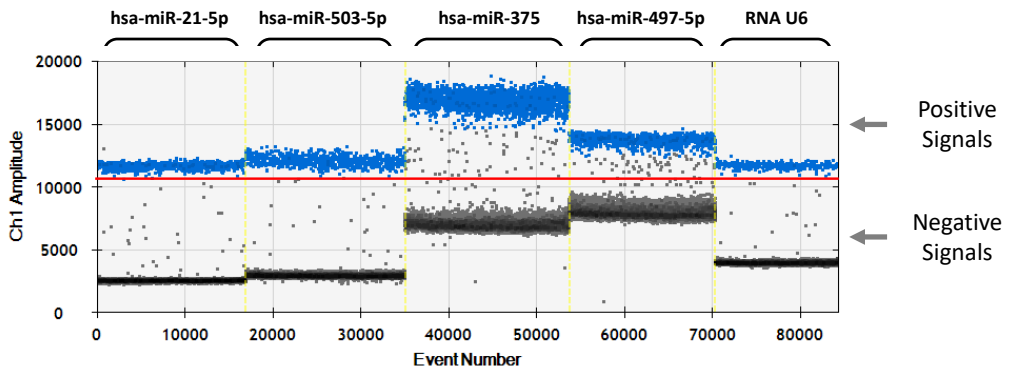

Figure 12. Representative images of ddPCR amplification signals obtained for all the selected miRNAs.

Overall, the data obtained confirmed the high-predictive value of the computational analyses previously performed. In particular, statistically significant data were obtained for all the miRNAs analyzed. Of these, hsa-miR-21-5p and hsa-miR-503-5p showed the strongest significant increase in tumor samples compared to adjacent normal controls ($p<0.0001$ for both miRNAs). Also the expression levels between tumor and control samples were statistically different considering hsa-miR-375 and hsa-miR-497-5p ($p=0.0073$ and $p=0.0064$, respectively) (Figure 13).

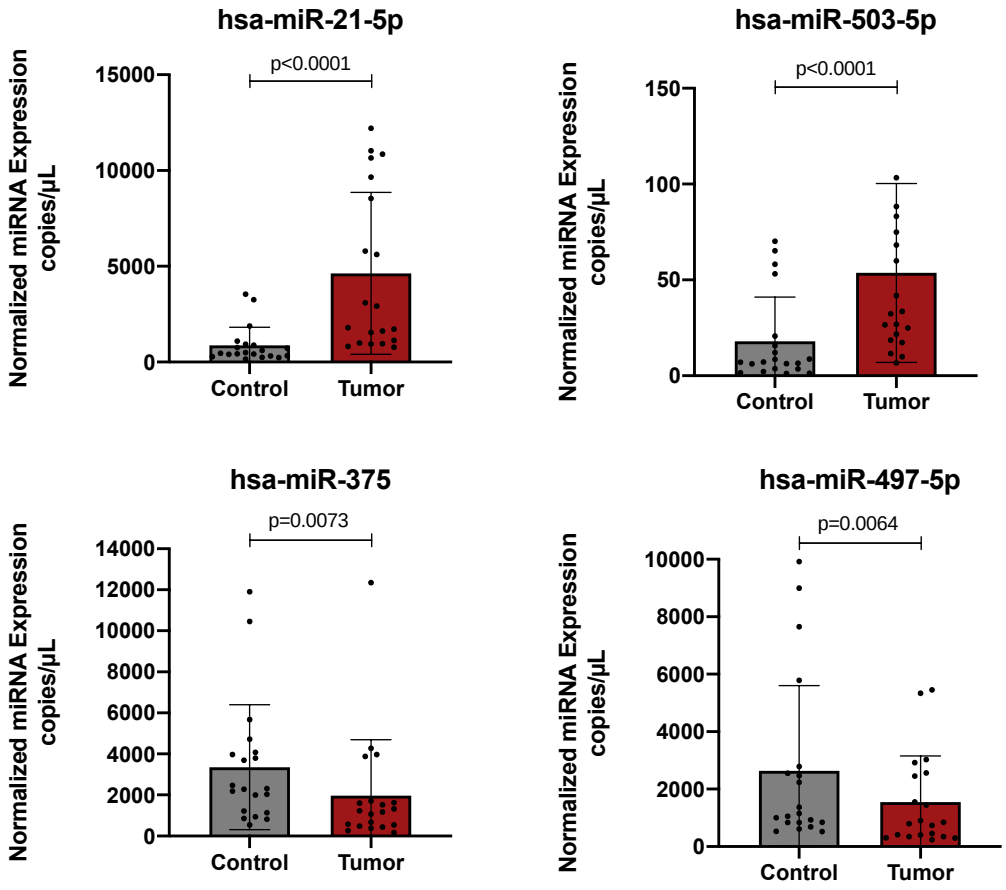

Figure 13. Tissue expression levels of hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p in FFPE samples of colorectal cancer and normal adjacent mucosa. Data were considered significant for $p<0.05$.

These data widely confirmed all the previous bioinformatics analyses, suggesting how the computational evaluation of miRNA expression data could be useful to identify novel biomarkers for the effective diagnosis of colorectal cancer.

To better evaluate the diagnostic potential of the four selected miRNAs, ROC analyses were performed. In this case, ROC analyses revealed a very good diagnostic value for hsa-miR-21-5p and hsa-miR-503-5p showing AUC value of 0.8925 ($p<0.0001$) and 0.8350 ($p=0.0003$), respectively (Figure 14). As regards the two down-regulated miRNAs, significant data were obtained for hsa-miR-375 showing a AUC of 0.7200 ($p<0.0173$), while a low AUC value was obtained for hsa-miR-497-5p (AUC=0.6550, $p=0.0935$) (Figure 14).

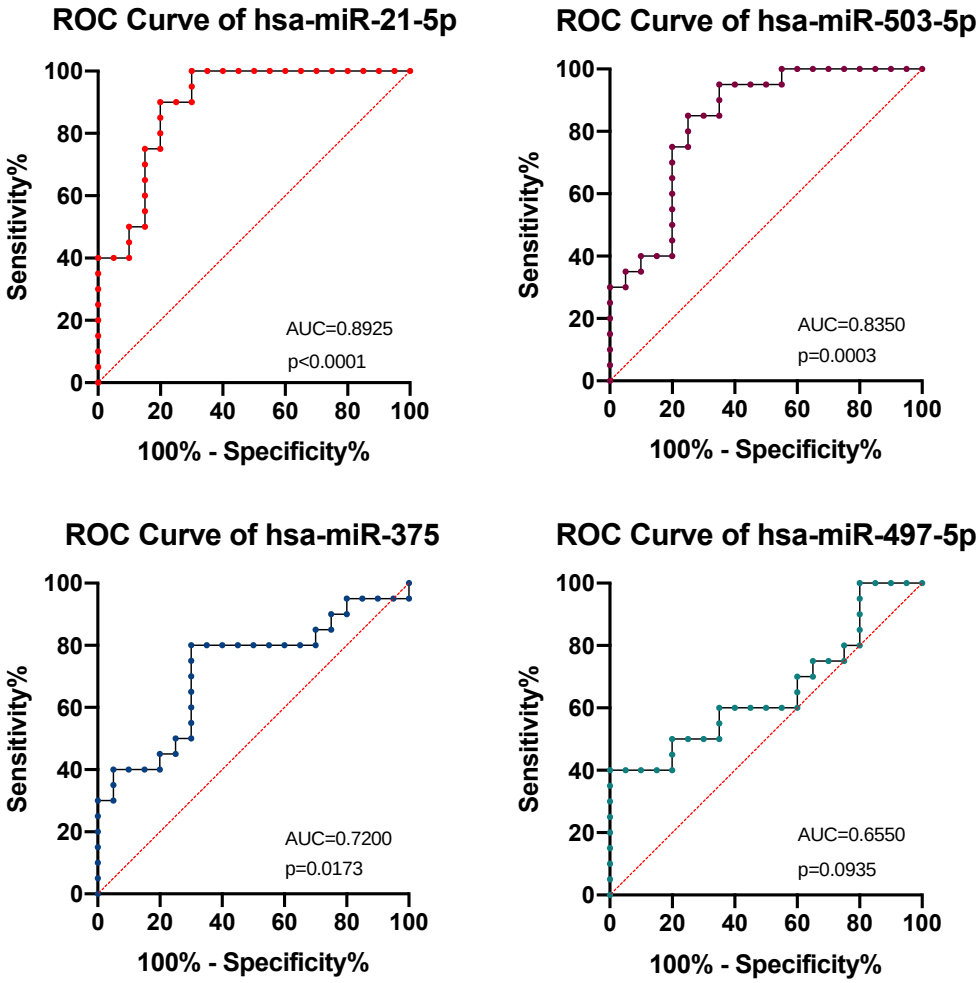

Figure 14. Diagnostic potential of the selected miRNAs according to the ROC analyses. Data were considered significant for $p<0.05$.

Overall, hsa-miR-21-5p showed a sensitivity of 90% and a specificity of 80%; hsa-miR-503 showed a sensitivity of 95% and a specificity of 75% while the down-regulated hsa-miR-375 had a sensitivity of 90% but a very low specificity of 25%. For hsa-miR-497-5p these analyses were not performed because of the non-significant data obtained.

To evaluate the diagnostic potential of the four selected miRNAs as circulating biomarkers for the early diagnosis of colorectal cancer, the expression levels of these miRNAs were also evaluated in liquid biopsy samples collected from 15 colorectal cancer patients and 15 healthy controls.

The ddPCR analyses performed on serum samples revealed significant results only for hsa-miR-21-5p and hsa-miR-497-5p which were significantly up-regulated and down-regulated in colorectal cancer patients compared to healthy

controls, respectively (p=0.408 and p=0.0164). As regards hsa-miR-503-5p and hsa-miR-375, the ddPCR analyses revealed, respectively, a trend of increment and a trend of decrement of these miRNAs in tumor samples, however, these data are not statistically significant (Figure 15).

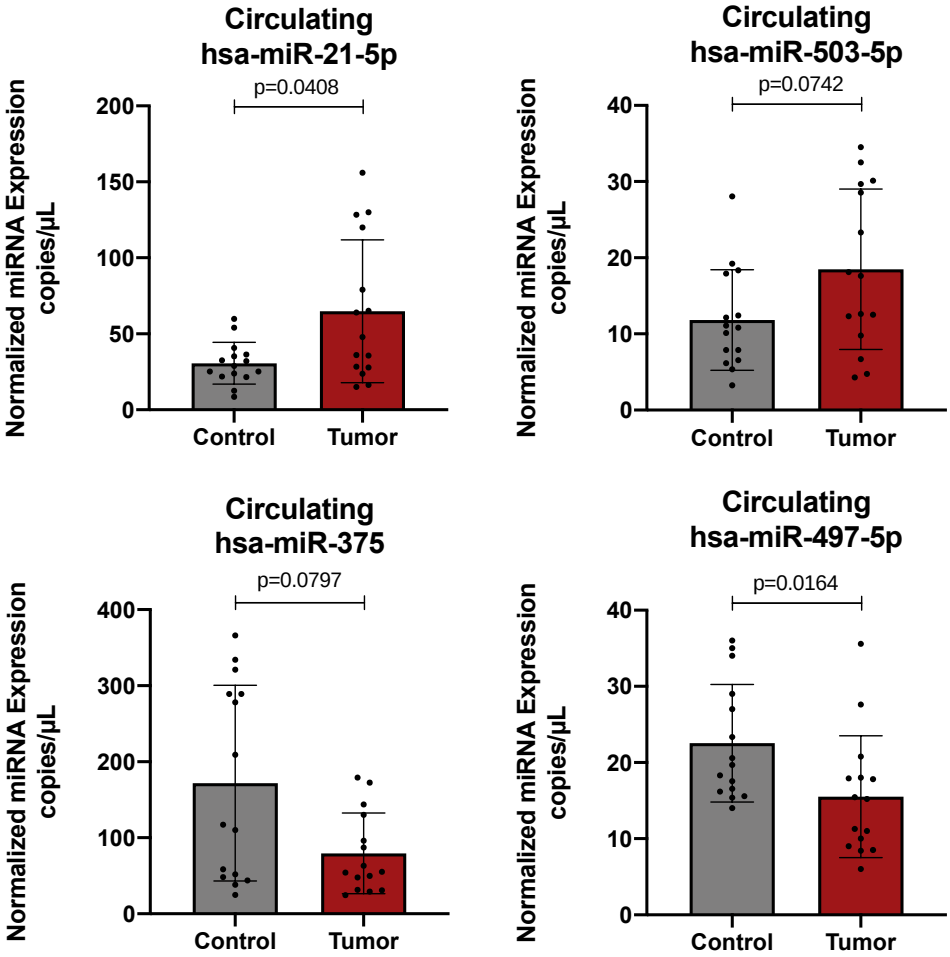

Figure 15. Circulating expression levels of hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p in liquid biopsy samples of colorectal cancer patients and healthy controls. Data were considered significant for $p<0.05$.

Of note, these latter results could be related to the low number of samples analyzed. Indeed, further analyses on a wider cohort of samples should be performed to improve the statistical power of our investigations.

Also for liquid biopsy samples and circulating miRNAs, ROC analyses were performed, however, weaker data were obtained. In particular, only the ROC curves obtained for hsa-miR-21-5p and hsa-miR-497-5p were statistically significant (AUC=0.7200, p=0.0401 and AUC=0.7556, p=0.0171), while lower

AUC values and no significant data were obtained for hsa-miR-503-5p and hsa-miR-375 (Figure 16).

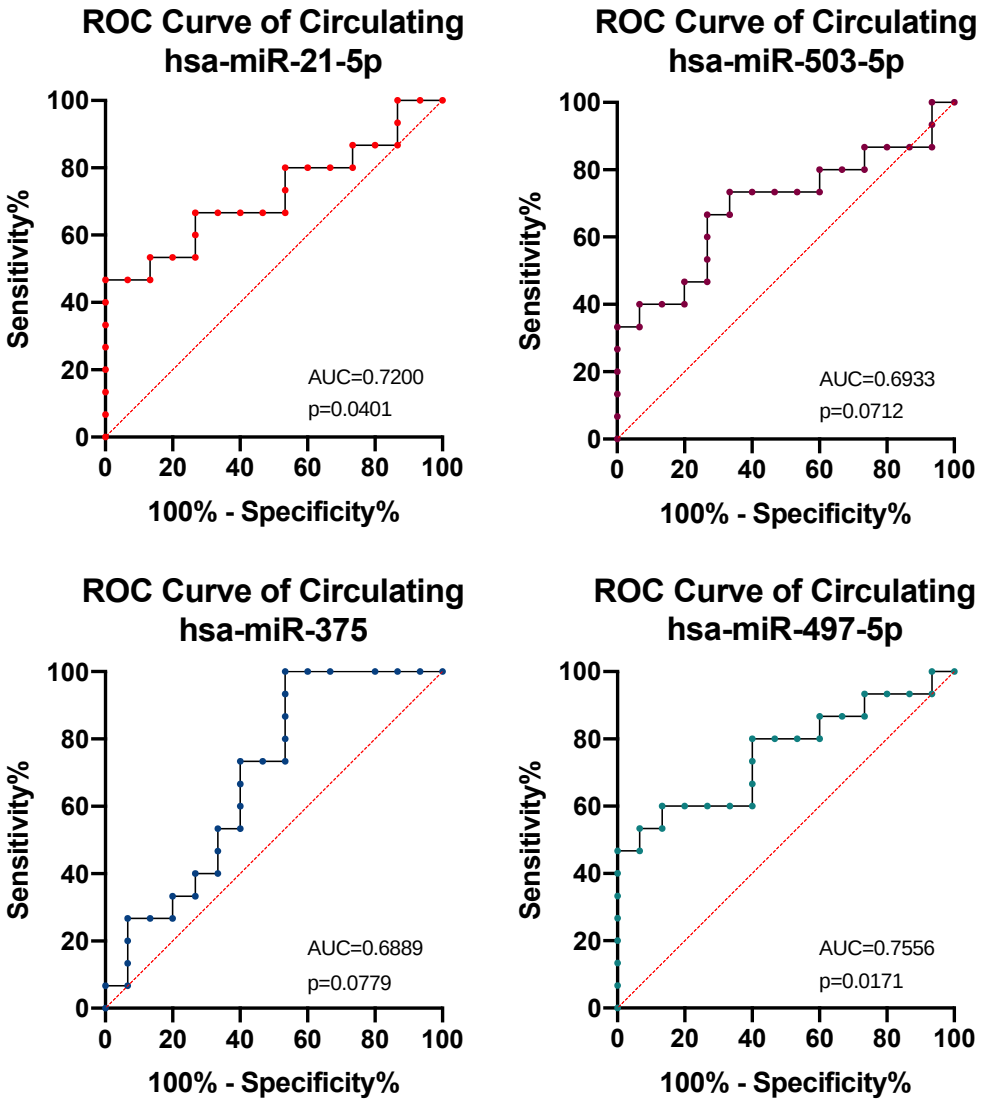

Figure 16. Diagnostic potential of the evaluation of circulating miRNAs according to the ROC analyses. Data were considered significant for $p<0.05$

Although statistically significant, the sensitivity and specificity rates for circulating hsa-miR-21-5p and hsa-miR-497-5p were lower than those observed for tissue miRNA expression levels.

All these molecular data collected for both FFPE and liquid biopsy samples revealed the good predictive value of the computational analyses performed and suggest how the concomitant evaluation of a miRNA signature could be useful to early detect the presence of a colorectal tumor, especially in those individuals at risk for this pathology. However, as already mentioned, these preliminary

results need further validation on a wider cohort of colorectal cancer patients, individuals at risk for this tumor and normal control in order to strengthen the statistical significance of the data here obtained. Indeed, a limitation of the study is the lack of clinical-pathological data of the patients enrolled in this study and the lack of samples collected during the follow-up of patients.

In this regard, further computational analyses performed on the miRNA expression data and clinical-pathological data contained in the TCGA COADREAD database were performed to establish also the prognostic value of the miRNAs here identified.

4.3 Further Bioinformatics Analyses and Prognostic Value of the Four Selected microRNAs

As demonstrated by the results obtained through the DIANA-mirPath analyses, the 19 selected miRNAs are able to alter the expression levels of a huge number of genes and molecular pathways. In addition, the ddPCR analyses confirmed that the miRNAs hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p are significantly dysregulated in colorectal cancer samples compared to normal controls. To further evaluate the genes and functions altered by these four miRNAs, other computational analyses were performed.

Through the miRTargetLink Human software, the genes with strong and weak interactions with the four validated miRNAs were identified. As regards the genes with strong and validated interaction with the four miRNAs, the miRTargetLink Human analysis revealed that these miRNAs were able to strongly bind and alter the expression levels of a total of ten different genes (Figure 17).

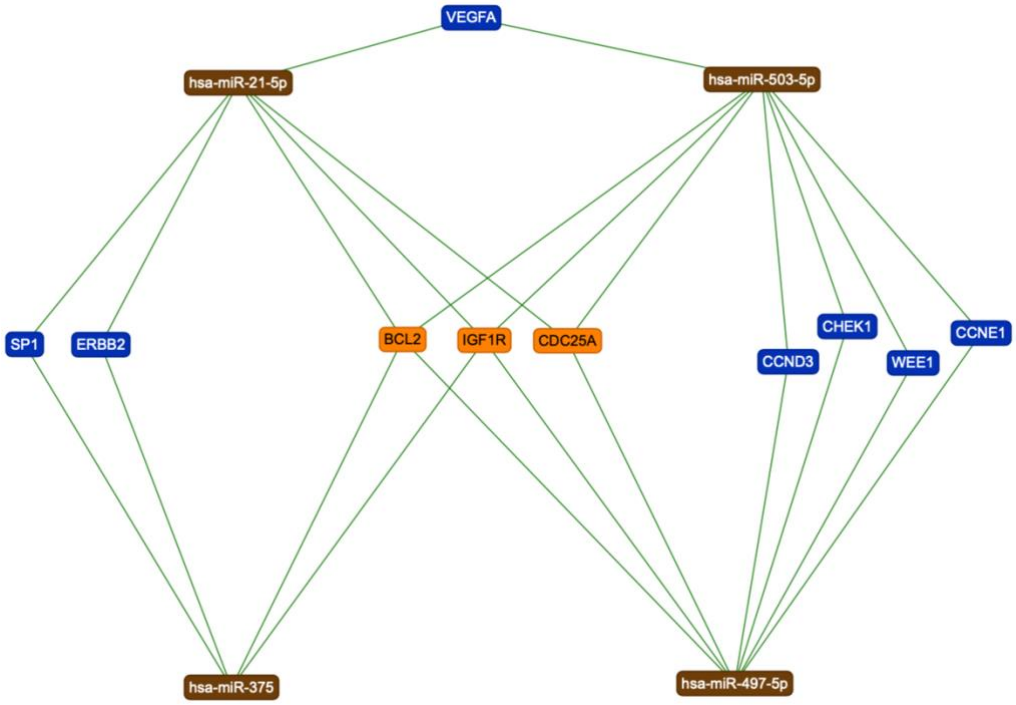

Figure 17. Network of genes with strong interactions with one or more of the four validated miRNAs according to the miRTargetLink Human analysis.

More in detail, three genes, i.e. BCL2, IGF1R and CDC25A were targeted by all the selected miRNAs, suggesting how the expression levels of these genes may be strongly modulated by epigenetic mechanisms, including miRNA expression. Interestingly, between the two down-regulated miRNAs hsa-miR-375 and hsa-miR-497-5p no genes were shared, while between the two up-regulated miRNAs hsa-miR-21-5p and hsa-miR503-5p only the VEGFA gene was in common. As regards the other genes identified, SP1 and ERBB2 were shared between hsa-miR-21-5p and hsa-miR-375, while CCND3, CHEK1, WEE1 and CCNE1 were in common between hsa-miR-503-5p and hsa-miR-497-5p. Therefore, the alteration of the expression levels of these latter genes may be the result of a fine regulation mediated by both up-regulated and down-regulated miRNAs.

Noteworthy, most of these ten genes identified are known to be involved in the alteration of key cellular mechanisms like proliferation and apoptosis and their dysregulation is already be associated with the development of tumors, including colorectal cancer.

The same miRTargetLink Human analysis of the four validated miRNAs was performed to identify the genes with weak interaction with the selected miRNAs.

This latter analysis revealed a more complex network, however, these interactions are only predicted and not validated therefore they cannot be accepted as verified and reliable (Figure 18).

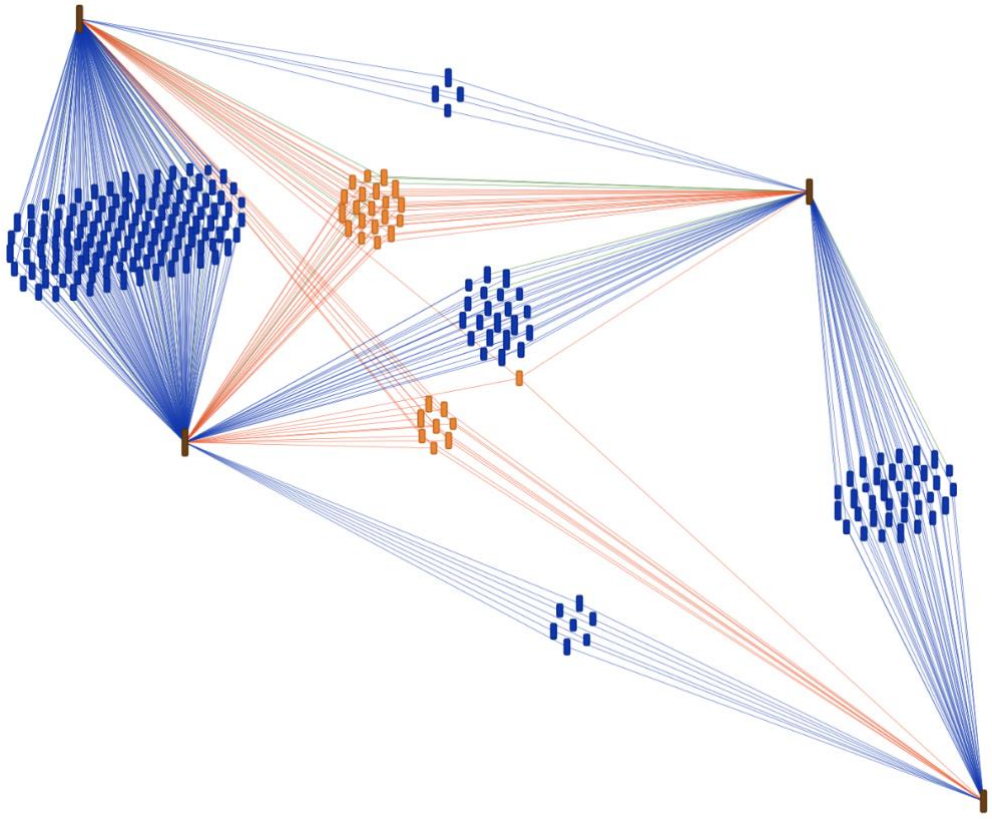

Figure 18. Network of genes with weak interactions with one or more of the four validated miRNAs according to the miRTargetLink Human analysis.

To further establish the protein-protein interaction between the ten genes strongly modulated by the four validated miRNAs the STRING analysis was performed. This further analysis confirmed the strong interconnections existing between these miRNA-modulated genes (Figure 19).

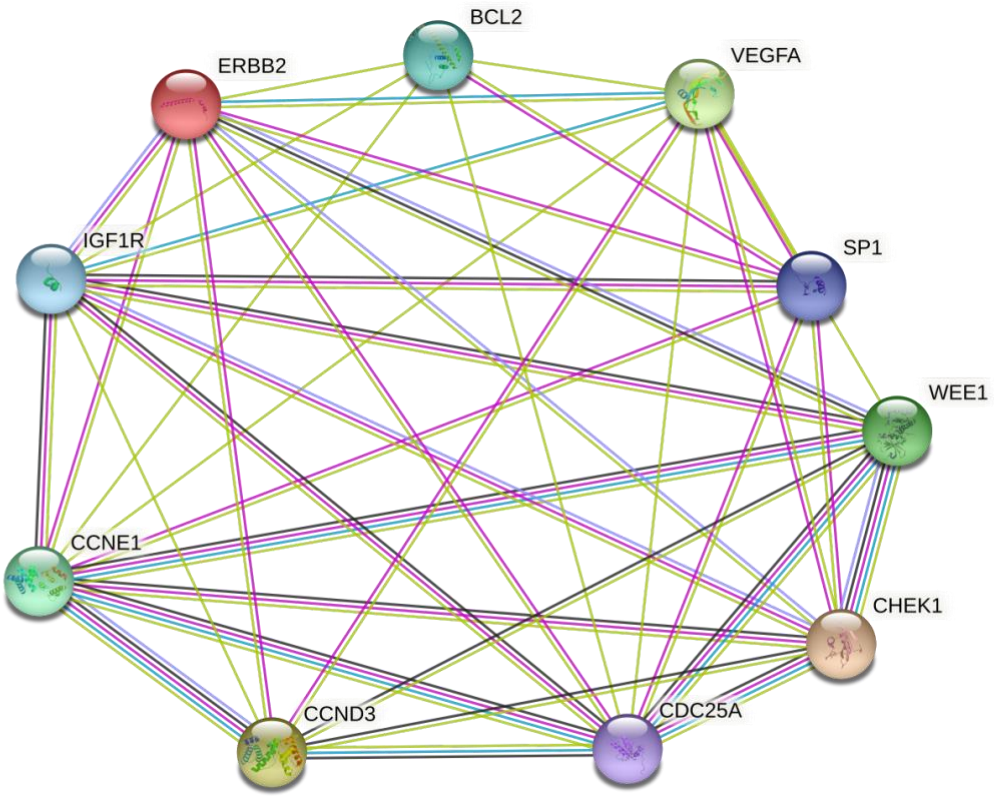

Figure 19. STRING protein-protein interaction network of the ten genes strongly modulated by the four validated miRNAs.

This further analysis highlighted how among the ten genes, WEE1, CHEK1, CDC25A, CCND3, CCNE1 and IGF1R were those with the more complex interactions. However, overall, all these genes are strongly interconnected each other.

A more in-depth analysis of the functional roles of these genes performed through GO Panther allowed to establish the molecular functions, the biological processes, the protein class and the molecular pathways where these ten genes are involved and thus the functions, processes and pathways that the four miRNAs are able to modulate.

As regards, the molecular functions, the majority of genes were involved in binding and catalytic activities (seven genes for both categories) while other functions were molecular function regulator (five genes) and molecular transducer activities (two genes) (Figure 20).

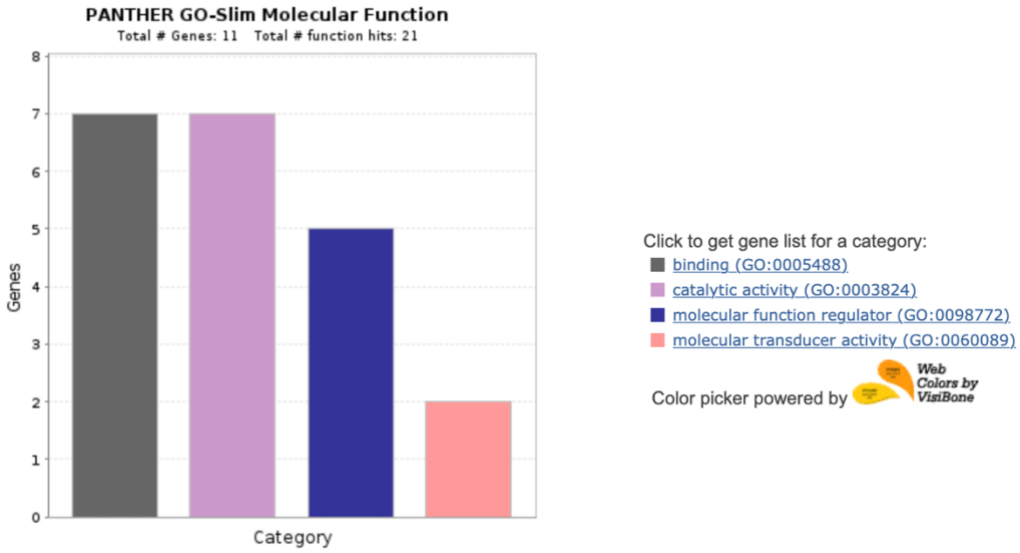

Figure 20. GO Panther analysis of the molecular functions of the ten genes targeted by the four validated miRNAs.

As regards the biological processes, the ten identified genes are involved in multiple processes. The majority of these genes are involved in biological regulation and cellular metabolic and signaling processes. Among the ten genes some were also involved in the response to stimuli and signaling (Figure 21).

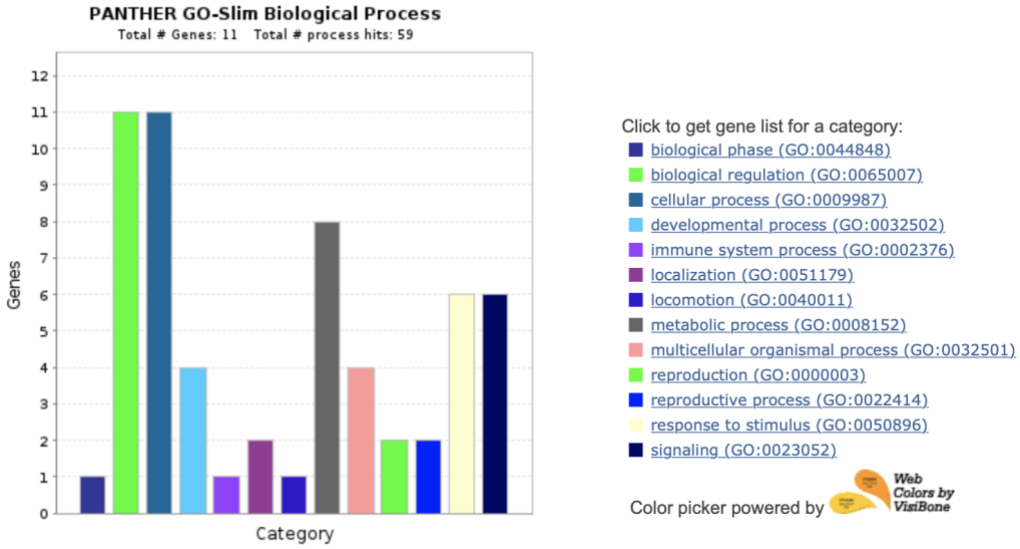

Figure 21. GO Panther analysis of the biological processes of the ten genes targeted by the four validated miRNAs.

By analyzing the protein class of the ten genes identified, it was observed that three are protein modifying enzymes, two are transmembrane signal receptors, two are protein-binding activity modulators, one is a gene-specific transcriptional regulator and one is a protein-binding activity modulator (Figure 22).

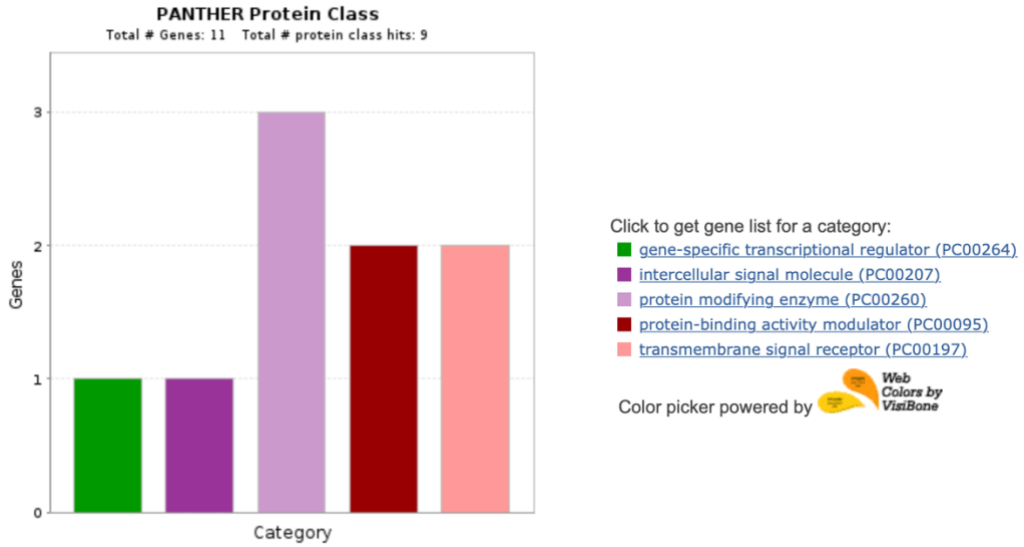

Figure 22. GO Panther analysis of the protein classes of the ten genes targeted by the four validated miRNAs.

Finally, the ten identified genes were analyzed according to their molecular pathways. This latter evaluation revealed as the majority of genes were involved in cell cycle, p53 pathway, gonadotropin-releasing pathway and CCKR signaling map (Figure 23).

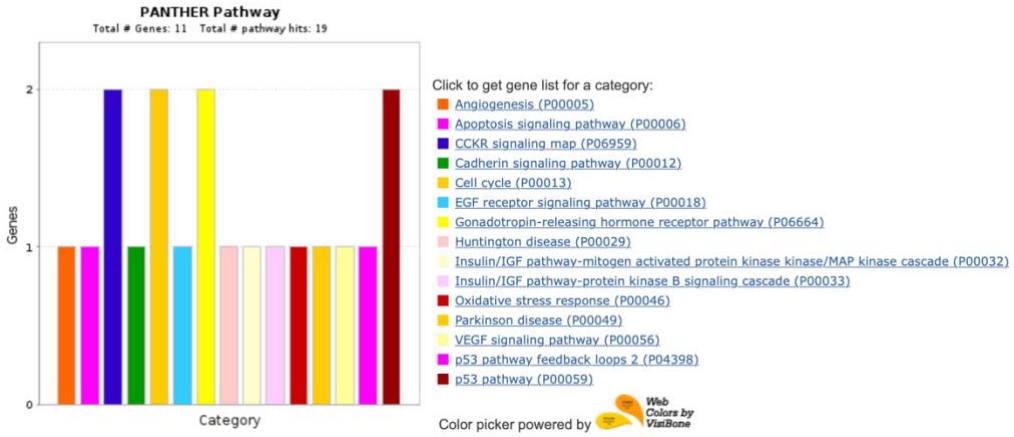

Figure 23. GO Panther analysis of the molecular pathways of the ten genes targeted by the four validated miRNAs.

Overall, all these analyses revealed that t1he four selected miRNAs are able to alter a plethora of cellular and molecular aspects through the strong regulation of only ten genes. These data suggest how epigenetic mechanisms may play a fundamental role in the molecular mechanisms underlying the development and progression of tumors.

To conclude the computational analyses on the functional roles of the four validated miRNAs, the dysregulation of these ten genes in colorectal cancer patients was evaluated by using the computational tool GEPIA.

GEPIA analysis revealed a strong and significant dysregulation for five out of the ten genes selected (Figure 24).

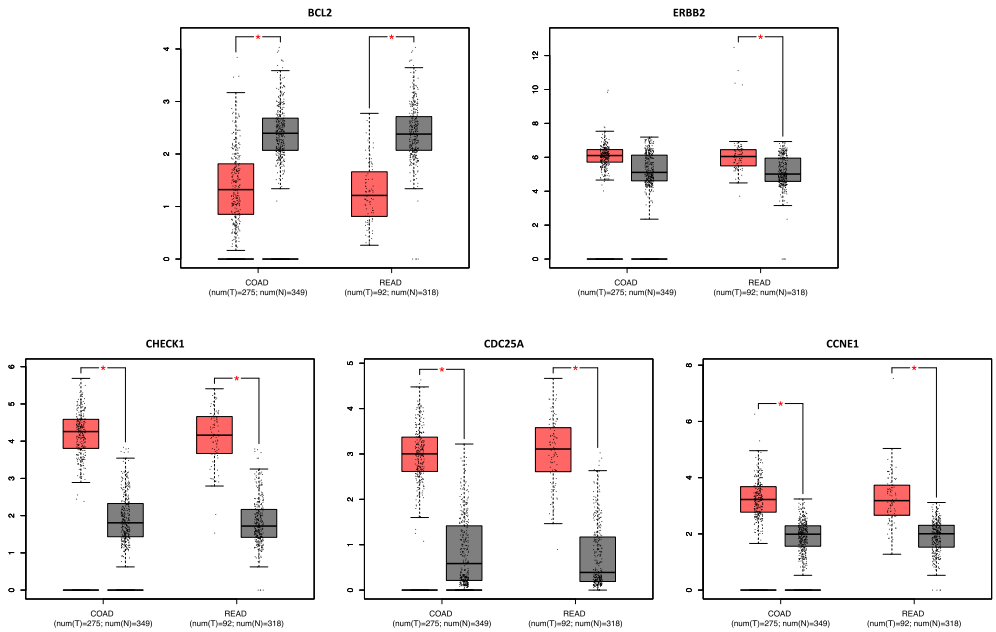

Figure 24. GEPIA analysis of the expression levels of the ten miRNA-targeted genes in colon (COAD) and rectal (READ) cancer patients compared to healthy controls according to the data contained in the TCGA database.

In particular, GEPIA analysis revealed that BCL2 expression levels were significantly reduced in both COAD and READ patients compared to healthy controls. In addition, a slight increment of ERBB2 expression levels was observed for READ patients but not for COAD ones. As regards CHEK1, CDC25A and CCNE1 the expression levels were significantly higher in both COAD and READ samples compared to the normal counterpart (Figure 24).

Of note, two of the three genes targeted by all the four miRNAs, i.e. BCL2 and CDC25A were both significantly dysregulated in colorectal cancer patients potentially as a result of different epigenetic mechanisms acting on the expression levels of these genes.

Finally, the OncoLnc analysis was performed to evaluate the prognostic significance of the four validated miRNAs hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-375 and hsa-miR-497-5p in correctly predicting the overall survival

rates of colorectal cancer patients. This further analysis confirmed that besides the previously validated diagnostic roles, the up-regulation of hsa-miR-21-5p and hsa-miR-503-5p as well as the down-regulation of hsa-miR-375 can be considered excellent prognostic biomarkers for patients with colon cancer as the dysregulation of these three miRNAs is significantly associated with a low overall survival (Logrank p-value=0.0304, Logrank p-value=0.03 and Logrank p-value=0.0196, respectively) (Figure 25).

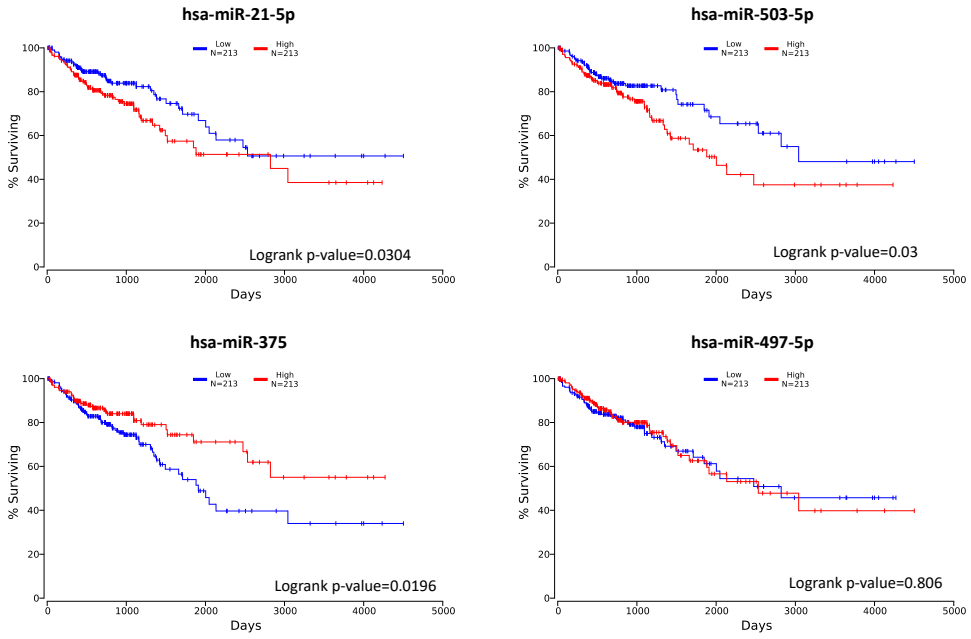

Figure 25. Prognostic value of the four validated miRNAs in colon cancer patients according to OncoLnc analysis.

The same OncoLnc analysis performed for patients with rectal cancers contained in the TCGA READ database demonstrated that only the down-regulated hsa-miR-375 can be considered an excellent prognostic biomarker to define the overall survival of rectal cancer patients (Logrank p-value=0.00152) (Figure 26).

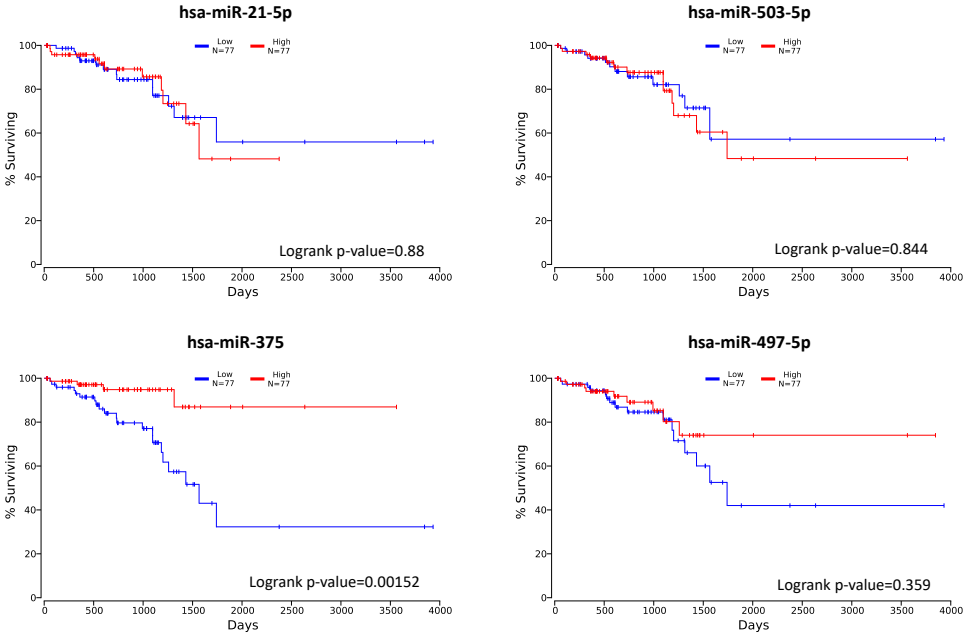

Figure 26. Prognostic value of the four validated miRNAs in rectal cancer patients according to OncoLnc analysis.

Of note, no significant data were obtained for the two up-regulated miRNAs and for the down-regulated hsa-miR-497-5p although a trend of better survival is observed for patients with high expression levels of this miRNA.

5. DISCUSSION

Although the development and progression of both surgical and pharmacological strategies for the treatment of colorectal cancer patients, this tumor still represents one of the most diagnosed worldwide and is included in the category of "big killer" tumor together with lung cancer, breast cancer, etc. [Xu W et al, 2016; Akhtar R et al, 2014]. Several studies have tried to identify novel biomarkers for this pathology, however, conflicting data have been generated on this topic [Monahan KJ et al, 2017; Chang LC et al, 2017; Diamandis EP, 2010]. Recently, different studies have obtained encouraging results about the diagnostic and prognostic potential of microRNAs (miRNAs), a class of small non-coding RNA, in different tumors. In particular, both computational and experimental studies have demonstrated the diagnostic potential of the analysis of the expression levels of both circulating and tissue miRNAs for oral cancer, breast cancer, uveal melanoma, etc. [Falzone L et al, 2020; Crimi S et al, 2020; Falzone L et al, 2019].

In particular, the discovery of circulating miRNAs stable and easily detectable in body fluids, prompted researchers worldwide in investigating the alterations of miRNAs expression levels in liquid biopsy samples in order to use these data as non-invasive and high-specific biomarkers for the prediction of the development of tumors [Baldassarre A et al, 2017; Thind A and Wilson C, 2016; Cho WC, 2010].

At present, different studies have investigated the role of miRNAs in colorectal cancer development, however, no conclusive results have been obtained and the studies available in literature do not consider the huge amount of bioinformatics data available on public omics databases like GEO DataSets or TCGA. In this scenario, no effective biomarkers are currently available for the early detection of colorectal cancer. Therefore, the analysis of miRNAs dysregulated in cancer patients may represent a good strategy for the early diagnosis and prognosis of different tumor types, including colorectal cancer.

Starting from these observations, in the present study we speculated the potential role of a bioinformatics approach to identify miRNAs with high diagnostic and prognostic significance in cancer development and progression [Falzone L et al,

2016; Hafsi S et al, 2016] as well as how high-sensitive technologies, like ddPCR, could be useful to precisely evaluate alterations in the expression levels of miRNAs in colorectal cancer patients or individuals at risk for this pathology. On these bases, an integrated computational analysis was preliminary performed taking into account all microRNA expression profiling datasets containing the miRNAs expression data of colorectal cancer samples and normal tissues in order to identify a panel of miRNAs potentially involved in the development and progression of colorectal cancer and thus potentially useful as biomarkers for this disease.

Specifically, the bioinformatics analyses were performed on ten microRNA microarray expression datasets selected from the GEO DataSets database by applying specific inclusion and exclusion criteria. The differential analyses performed between the expression levels of miRNAs in colorectal cancer samples compared to the expression levels of the same miRNAs in healthy tissues allowed us to identify a set of miRNAs significantly dysregulated in colorectal cancer. By merging the data generated from each dataset, a set of ten up-regulated miRNAs and ten down-regulated in colorectal cancer were identified. Subsequently, one miRNA, the up-regulated miRNA hsa-miR-1308, was excluded from the further analyses as it is not a miRNA but a fragment of a tRNA.

These preliminary computational analyses allowed us to identify different miRNAs known to be involved in the development and progression of tumors. Among these, the most significant and up-regulated miRNAs there were hsa-miR-18a-5p, hsa-miR-21-5p and hsa-miR-503-5p while among the most down-regulated were the hsa-miR-133b, hsa-miR-375 and hsa-miR-497-5p. All these frequently modulated miRNAs in colorectal cancer are already described in literature as miRNAs strongly involved in this tumor highlighting the consistency and effectiveness of the bioinformatics analyses performed [Wang X et al, 2017; Wei R et al, 2017].

Further computational analyses revealed that these 19 miRNAs were able to modulate a plethora of genes known to be involved in different cellular and molecular pathways responsible for the neoplastic transformation of colon cells.

In particular, through COSMIC the most altered genes were identified, while mirDIP analysis showed an overall medium-high interaction between the selected miRNAs and the altered genes in CRC.

In particular, the gene target analysis revealed that the miRNAs hsa-miR-223-3p, hsa-miR-195-5p and hsa-miR-497-5p showed the highest interaction levels among all selected miRNAs, with a particular specificity for the APC, KRAS, KMT2C and ZFHX3. Interestingly, the interaction values of the hsa-miR-195-5p and hsa-miR-497-5 were almost completely overlapping (9 of 10 gene interactions were the same). Of note, these 2 miRNAs were organized in a genomic cluster at the chromosome 17 in position p13.1 and this analysis suggested the same functional roles of these 2 miRNAs [Flavin RJ et al, 2009].

These 3 miRNAs were also already correlated to CRC and reported in literature. The authors described the diagnostic value of hsa-miR-223 and its role in CRC progression [Li ZW et al, 2014] and the importance of the miR-497∼195 cluster that were down-regulated in CRC [Yang M et al, 2017; Qiu Y et al, 2016; Zhang X et al, 2016].

Finally, the DIANA-mirPath analyses demonstrated an effective role of the selected miRNAs in the modulation of several colorectal cancer pathways highlighting their possible involvement in CRC development. Among the most targeted genes there were AKT, BCL2, Cyclin family (CCND1, CCND2, CCND3, etc), Cyclin-dependent kinase family (CDK4 and CDK6), EGFR, MAPK family, TP53, VEGFA, PIK3 family, etc. known to be involved in the development of colorectal cancer [Lin SH et al, 2017; Wee P et al, 2017]. Overall, the DIANA-mirPath analyses showed that the most altered pathways were MAPK and PI3K/Akt signaling pathways and Pathways in cancer.

Taking into account all these preliminary bioinformatics results, the up-regulated miRNAs hsa-miR-21-5p and hsa-miR-503-5p and the down-regulated miRNAs hsa-miR-375 and hsa-miR-497-5p are able to target the highest number of genes within the aforementioned pathways. All these miRNAs are well described in the literature and have been related to several cancer types. Therefore, these four miRNAs were selected for the further validation analyses performed on FFPE

samples and liquid biopsy samples obtained from colorectal cancer patients and healthy controls.

To validate the diagnostic potential of the four selected miRNAs, the high-sensitive ddPCR amplification system was used. ddPCR was chosen for our analysis because it represents the best platform available for the analysis of samples expressed with low amounts as circulating miRNAs or for samples with poor quality material like FFPE tissue samples which are often degraded by the fixation processes.

Through the use of ddPCR we demonstrated that the expression levels of hsa-miR-21-5p and hsa-miR-503-5p were significantly increased in colorectal cancer FFPE samples compared to the normal adjacent mucosa while the expression levels of hsa-miR-375 and hsa-miR-497-5p were significantly down-regulated in tumor tissues as predicted by our computational approach.

Although the expression levels of the four selected miRNAs were strongly validated in FFPE tissue samples, weaker results were obtained for the liquid biopsy samples. Indeed, a statistical increment was observed only for hsa-miR-21-5p while only a trend of increment (non-significant) was observed for hsa-miR-503-5p. Similarly, only hsa-miR-497-5p showed a significant decrement of the circulating levels in tumor patients ($p<0.0164$) while a non-significant trend of decrement was observed for hsa-miR-375.

According to the results obtained for both FFPE and liquid biopsy samples, ROC analyses revealed a good diagnostic potential for the hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375 when investigated in tissues samples, while only a limited diagnostic value was observed for the same miRNAs when investigated in liquid biopsy samples.

To further establish the functional roles of the four validated miRNAs, further computational analyses were performed. In particular, miRTargetLink Human and STRING and GO Panther analyses allowed the identification of ten genes strongly modulated by the four miRNAs and able to interact each other thus regulating different molecular and biological processes. Among these ten genes, some are already known to be involved in colorectal cancer development and progression. Indeed, BCL2 is known to be down-regulated in colorectal cancer

with a worse prognosis [Ramesh P and Medema JP, 2020]. Similarly, ERBB2 and VEGFA are both involved in the aggressiveness of colorectal cancer through the increment of proliferation rate and angiogenesis respectively [Ross JS et al, 2018; Cui W et al, 2017].

Overall, these further analyses revealed as miRNAs are directly involved in the regulation of the neoplastic processes underlying the development of tumors through the dysregulation of key oncogenes and tumor suppressor genes widely described in literature.

Finally, to better elucidate the prognostic potential of the four validated miRNAs, the TCGA miRNA expression and survival data were analyzed by using the prediction tool OncoLnc. These further analyses revealed that the circulating levels of hsa-miR-375 can be used as a reliable prognostic biomarker to establish the overall survival of patients. Similarly, the tissue levels of hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-375 can predict the overall survival rate of patients at the diagnosis.

Overall, the results of the present study confirmed the bioinformatics results previously obtained. Indeed, despite the low number of samples analyzed, all the four miRNAs were confirmed as good diagnostic biomarkers and prognostic biomarkers (except for hsa-miR-497-5p). Therefore, this study represents the starting point for the adoption of ddPCR for the effective and sensitive analysis of miRNA expression levels both in tissue and liquid biopsy samples. Thus, this strategy could be applied for the non-invasive early diagnosis of colorectal cancer for individuals at risk for this tumor.

6. CONCLUSIONS

In conclusion, the present study allowed us to identify a panel of miRNAs significantly dysregulated in colorectal cancer and potentially used as diagnostic and prognostic biomarkers for this tumor. Through different computational analyses, four of these miRNAs computationally identified were selected for the validation experiments performed on FFPE samples and liquid biopsy samples. The validation experiments performed through ddPCR revealed the high diagnostic potential of the predicted up-regulated hsa-miR-21-5p and hsa-miR-503-5p and for the down-regulated hsa-miR-375, while weaker results were obtained for hsa-miR-497-5p. Overall, the evaluation of these miRNAs gave more reliable results when performed on FFPE tissue samples compared to liquid biopsy samples. However, the evaluations of this miRNA signature in both types of specimens could be helpful for a correct diagnosis of colorectal cancer.

Besides the validated diagnostic role, further computational analyses demonstrated the prognostic value of the selected miRNAs and in particular of hsa-miR-375 and hsa-miR-21-5p.

Overall, the results obtained in the present study represent the starting point on which to develop confirmatory studies carried out on a wider cohort of patients. Indeed, limitations of the study are the low number of samples analyzed and the lack of clinical-pathological data of the patients enrolled in this study and the lack of samples collected during the follow-up of patients. However, despite these limitations, the statistically significant results obtained pave the way to the development of novel diagnostic strategies based on the use of ddPCR and liquid biopsy for the analysis of miRNAs used as diagnostic and prognostic biomarkers for the management of colorectal cancers.

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