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Coordinator: Prof. Giancarlo Vecchio

"New biomolecular prognostic and predictive factors of response to treatments in colo-rectal cancer"

Giuseppina Della Vittoria Scarpati

University of Naples Federico II
Dipartimento di Biologia e Patologia Cellulare e Molecolare
"L. Califano"

Administrative Location

Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano"
Università degli Studi di Napoli Federico II

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Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore", CNR, Naples, Italy

Italian Faculty

Giancarlo Vecchio, MD, Co-ordinator
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Albert Einstein College of Medicine of Yeshiwa University, N.Y., USA
Luciano D'Adamio, MD
Nancy Carrasco, MD

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1 INTRODUCTION

1.1 Colorectal cancer

Colorectal cancer is the third most common cancer worldwide and the second leading cause of cancer-related death in the Western world. Approximately 75% of patients with colorectal cancer present with localised disease, whereas metastatic disease accounts for 25% of newly diagnosed patients. Systemic treatment is based on cytotoxic agents and monoclonal antibodies targeting epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). In the last decade, the optimal use of these agents in patients with metastatic disease has dramatically improved median survival from 6 to 24 months. However, despite all these therapeutic advances, the prognosis of patients with metastatic colorectal cancer remains poor, and, apart from those having surgery of isolated metastases, long-term survival is very low.

In the adjuvant setting, 5-fluorouracil- and oxaliplatin based chemotherapy has demonstrated a significant improvement in disease-free and overall survival, particularly in patients with resected stage III disease. The quantitative benefit of adjuvant therapy in patients with stage II colorectal cancer is less

important and remains a matter of controversy. However, despite this strategy, almost 40% of patients diagnosed initially with localised disease will develop recurrences. Therefore, there is a need to better identify these patients at risk and to adapt to them what we could consider optimal treatment. Anatomic criteria defined by Dukes almost 80 years ago are currently the basis of the tumour/node/metastasis (TNM) staging system upon which most clinical decisions concerning postoperative treatment are based.

However, some changes are expected to occur in the molecular medicine era. A high number of articles are reporting on the importance of several molecular markers, either predictive or prognostic, to help clinicians make better decisions with cancer patients. In light of this challenge, researchers are attempting to determine a more rational and pathogenic approach to cancer therapy. Markman et al. provide a well-written and comprehensive review aiming to highlight the prognostic and predictive biomarkers in colorectal cancer. An extensive amount of information is being published on many different mechanisms that may have therapeutic interest in colorectal cancer, but the important point is how to apply them properly in clinical practice. Improved understanding of cancer biology and advances in

biotechnology bring us closer to the concept of individualised treatment. A key component of this new paradigm is development of biomarkers that can guide application of new or even existing treatments. This requires a deep understanding of the relationship between biomarker and treatment effect.

Some recently published randomised studies are also designed to assess the importance of specific biomarkers. In others, the authors attempt to justify a retrospective assessment of certain biomarkers.

Nowadays, KRAS status testing is required prior to initiation of anti-EGFR antibodies.

Due to the complexity of the EGFR signalling system, it is likely that predictive algorithms will be developed for metastatic colorectal cancer, thus adding other molecular biomarkers. For instance, combining KRAS mutational status with the presence of b-Raf proto-oncogene serine/ threonine-protein kinase (BRAF) and/or phosphoinositide- 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutations and phosphatase and tensin homolog (PTEN) loss of function might identify additional patients who are unlikely to respond to treatment with EGFR-targeted monoclonal antibodies.

However, these additional markers require further validation before they can be incorporated into clinical practice.

Further work is also required to explore potential early markers of response that can be incorporated into the design of future prospective clinical trials and guide therapeutic decisions regarding continuation of treatment in individual patients. In 1990, Fearon and Vogelstein presented their well-known model for the genetic basis of colorectal neoplasia. They proposed that "identification of the genetic alterations present in tumours may provide a molecular tool for improved estimation of prognosis in patients with colorectal cancer"

Nowadays, almost two decades later, we still have much to learn about the prognostic or predictive value of these and others "colorectal cancer biomarkers".

Molecular profiling of tumours may certainly let us identify patients who are more likely to benefit from a specific therapy. This would stimulate clinicians to tailor treatment according to an individual patient and/or tumour profile, converting the so-called personalised or tailored therapy into a reality. New methodologies such as microarray-based gene expression profiling, proteomic profiling, comparative genomic

hybridisation analysis and metabolomics would allow tumour samples to be profiled on a global scale.

The most frequently used genomewide approach is DNA microarray profiling. One platform is the Oncotype DX colon cancer test being used to profile gene expression. Molecular alterations are used to redesign the taxonomy of solid tumours by moving it from a histologically to a genetically based level.

Parallel development of predictive molecular and clinical markers is paramount to achieve the best outcomes from targeted treatments, and KRAS is so far the only validated predictive molecular marker in colorectal cancer for EGFR-directed monoclonal antibodies. In the future, we expect to employ a personalised or tailored therapy that will become a reality, with less toxicity and superior efficacy.

This will become a reality only if we are able to improve our molecular knowledge, we can consistently validate these new biomarkers and we can eventually apply them in our clinical decisions.

Clinical and translational research that is in progress will hopefully help to provide the much promised hope of personalised medicine in the management of colorectal cancer.

We can foresee that in some years, the treatment for advanced colon cancer will be based upon molecular features.

1. 2 MicroRNAs

MicroRNAs (miRNAs) are small regulatory RNA molecules functioning to modulate gene expression at the post-transcriptional level, and playing an important role in the control of many biological processes, such as cellular development, differentiation, proliferation, apoptosis and metabolism. They are implicated in pathogenesis, diagnosis and therapeutic aspects of viral infections, cardiovascular disease, and neurological and muscular disorders. Data recently published suggest that the expression of certain genes can be more dependent on the levels of regulatory miRNAs than on the levels of messenger RNAs that encode the proteins. RNA mediated gene silencing pathways have essential roles in development, cell differentiation, cell proliferation, cell death, chromosome structure and virus resistance. Moreover, studies from the last three years have demonstrated that there is altered expression of miRNA genes in many human malignancies.

The DNA sequence that codes for a miRNA gene is longer than the miRNA. This DNA sequence includes the miRNA sequence and an approximate reverse complement. When this DNA sequence is transcribed into a single-stranded RNA molecule, the miRNA sequence and its reverse-complement base pair to form a

double stranded RNA hairpin loop; this forms a primary miRNA structure (pri-miRNA) that contains a stem-loop structure of about 80 bases.

Pri-miRNAs are processed in the nucleus by the RNase III enzyme Drosha and DGCR8/Pasha, which excises the stem-loop to form the pre-miRNA. Pre-miRNAs are exported from the nucleus by Exportin-5, a carrier protein. In the cytoplasm another RNase III enzyme, Dicer, cuts the pre-miRNA to generate the mature microRNA as part of a short RNA duplex. The RNA is subsequently unwound by a helicase activity and incorporated into a RNA induced silencing complex (RISC).

The function of miRNAs appears to be in gene regulation. For that purpose, a miRNA is complementary to a part of one or more messenger RNAs (mRNAs), usually to a site in the 3' UTR. The annealing of the miRNA to the mRNA then inhibits protein translation, but sometimes facilitates cleavage of the mRNA. In such cases, the formation of the double-stranded RNA through the binding of the miRNA triggers the degradation of the mRNA transcript through a process similar to RNA interference (RNAi), though in other cases it is believed that the miRNA complex blocks the protein translation machinery or otherwise prevents protein translation without causing the mRNA to be degraded.

miRNAs may also target methylation of genomic sites which correspond to targeted mRNAs.

Ten thousand miRNAs have been discovered in various organisms. Generally, each miRNA is thought to regulate multiple genes, hundreds of miRNA genes are predicted and several hundreds have been already cloned and sequenced from C.elegans, Drosophila, Arabidopsis, mice and human. The large number of miRNAs and homologous sequences of many miRNAs among organisms suggest that these RNAs might constitute an abundant and conserved component of the gene regulatory machinery.

There are three groups of miRNA genes according to their genomic location:

1. Intronic miRNA in protein coding transcription units
2. Intronic miRNA in noncoding transcription units
3. Exonic miRNA in noncoding transcription units

It was initially thought that most miRNA genes are located in intergenic regions. A recent analysis of miRNA gene locations and transcription units witch involved combining genome assemblies and expressed sequence tag databases demonstrated more than 70% of mammalian miRNA genes are located in defined transcription units. Moreover, two thirds of miRNA genes are found in the introns in the sense orientation.

Interestingly miRNAs can be also present in either an exon or an intron depending on the alternative splicing pattern.

Human miRNA genes are located in all chromosomes except Y chromosome and they are nonrandomly distributed in the human genome. Approximately 50% of known human miRNAs are found in clusters and they are transcribed as polycistronic primary transcripts. There are usually two or three genes per cluster and the largest cluster at 13q31 is composed of seven genes. A cluster can contain miRNAs related to each other, suggesting that it is a result of gene duplication, or contains unrelated miRNAs. Such clustered miRNAs can be functionally related by targeting the same gene or different genes in the same metabolic pathway. It is possible that even in cases where clustered genes have no sequence homology, they may share functional relationships.

1.3 MicroRNAs and cancer

Many studies showed that miRNAs are aberrantly expressed in cancer, suggesting their role as a novel class of oncogenes or tumor suppressor genes. The findings that miRNAs have a role in cancer are supported by the fact that about 50% of miRNA genes are localised in cancer-associated genomic regions or in fragile sites. Regulation mediated by these genes has possibly a large impact on gene expression because, according to computional predictions, a single miRNA can target many genes. Many authors have reported that each cancer tissue has a specific microRNA "*signature*" and microRNA based cancer classification is a very effective and potential tool. First evidence of involvement of miRNAs in cancer came from molecular studies characterizing the 13q14 deletion in human chronic lymphocytic leukemia (CLL). Deletion of the 13q14 region occurs in more than half cases of B cell chronic lymphocytic leukemias and also in 50% of mantle cell lymphomas, in 16–40% of multiple myelomas and in 60% of prostate cancers, suggesting that tumor suppressor(s) gene(s) at 13q14 are involved in the pathogenesis of these tumors. Calin et al. (2002) have shown that two miRNA genes are located at 13q14.3 within a 30-kb region of minimal loss in CLL between two exons of the

LEU2 gene. Both of these genes, miR-15a and miR-16-1, are down-regulated in more than 60% of CLL cases (detected using Northern blot analyses). A very similar cluster (miR-15b, miR-16-2), but with a different promoter, was found on chromosome 3q25–26.1. It seems that these miRNAs are less intensivelly expressed in normal cells but may play a role in the cases of 13q14 deletions. Putative target of miR-16 is the arginyl-tRNA synthetase gene (RARS) because it has a homology of 85% on the 20-nt overlap and the levels of expression of the RARS gene correlate with the levels of expression of miR-16. Another evidence about the target gene for miR-16 came during the studies of pituitary adenomas, where miR-15a and miR-16-1 are expressed at lower levels as compared to normal pituitary tissue and their expression inversely correlates with RARS expression. Cimmino et al. (2005) have demonstrated a different possible target for miR-15a and miR-16-1, whose expression is inversely correlated to BCL2 expression in CLL and they have uncovered that both miRNAs negatively regulate BCL2 at a post-transcriptional level. Moreover, in a leukemic cell line model BCL2 repression by these microRNAs induces apoptosis. Deregulation of antiapoptotic BCL2 in CLL cells seems to be a key event in cancerogenesis. Recently, it became possible to

analyze the entire ***miRNome*** by microarrays containing all known human miRNAs. The use of miRNA microarrays made possible to confirm miR-16 deregulation in human CLL and also recognize miRNA expression signatures associated with defined clinicopathologic features. miR-16-1 and miR-15a , which were previously reported to be down-regulated in the majority (68%) of CLL cases by Northern analysis, were found to be expressed at low levels in 45% (miR-16-1) and in 25% (miR-15a) of CLL samples. These findings, that down-regulation of miR-16-1 and miR-15a expression correlates with allelic loss at 13q14, can be important for clinical classification of CLL. Patients with a normal karyotype or deletion of 13q14 as the sole genetic abnormality have a better prognosis than those with a complex karyotype or frequent deletion of 11q23 or 17p13. Expression profiling of miRNAs in human B-CLL identified significant differences in miRNome expression between CLL samples and normal CD5+ B lymphocytes. At the top of list of differently expressed miRNAs are several miRNAs located exactly inside fragile sites. In some miRNA genomic clusters all members are aberrantly regulated. In others only some members were abnormally expressed such as the largest known miRNA cluster - miR-17-92. Two miRNA expression clusters in CLL samples that

associate with the presence (20% as a cutoff) or absence of Zap-70 expression could be identified. ZAP-70 is a tyrosine kinase and low level of its expression is a predictor associated with good prognosis. Moreover, five differentially expressed miRNAs distinguish CLL samples that express unmutated IgVh locus from those that express mutated IgVh locus – a favorable prognostic factor. A signature composed of 13 microRNAs could well discriminate between a group of CLL samples that expresses ZAP-70 and unmutated IgVh (patients with worse prognosis) and the group that has no expression of ZAP and mutated IgVh (patients with better prognosis). Furthermore, members of the 13-member prognostic signature can well differentiate patients with a short interval from diagnosis to initial treatment (treatment begins with the development of the symptomatic or progressive disease) from patients with a longer interval. To summarize, the miRNA expression profile is associated with progression in CLL and can serve as a possible prognostic marker.

1.4 miRNAs in Colorectal cancer

Studies in the field of biomedicine have identified the specific miRNAs in colonic tissue and serum that might be used to screen for the presence of adenomas and colorectal cancers, and also to help predict disease recurrence. Adenomas (polyps) are precursors of most colorectal cancers and the progression of these lesions to cancer is a multistep process that involves different sequential DNA aberrations and changes in gene expression. Higher expression levels of miR-21 in adenomas and carcinomas relative to normal surrounding colonic tissue suggest that this represents an early cellular event in the progression to cancer. PTEN is associated with inhibition of cell invasion by blocking the expression of extracellular matrix metalloproteases, and miR-21 promotes cell migration and invasion by targeting the PTEN gene. Another pathway of metastasis was recently described for colorectal cancers in which miR-21 promotes intravasation, invasion and metastasis by downregulating the Pdcd4 gene. Overexpressed miRNAs such as miR- 20, miR-21, miR-17-5p, miR-15b, miR-181b, miR-191 and miR-200c have been implicated in colorectal cancer tissues. These tumour promoter miRNAs function by targeting and inhibiting different tumour

suppressor genes such as E2F transcription factor 1, tropomyosin 1, phosphatase and tension homologue gene (PTEN) and programmed cell death gene 4 (Pdcd4). Lower levels of mature miRNAs such as miR-34a, miR-126, miR- 143, miR-145 and miR-342 are also found in colorectal cancers, suggesting that they act as tumour suppressor miRNAs. The loss of such miRNAs may lead to overactivity of oncogenes. Besides identifying different cancer-related miRNAs, scientists are working to identify their target genes, messenger RNAs and receptors. Such identification will lead to further studies about their role in therapeutic manipulation and cancer treatment. For example, the putative identified targets of miR-145 are transforming growth factor receptor II and insulin receptor substrate 1 (IRS-1). The IRS-1 transduces mitogenic, antiapoptotic and anti differentiation signal that, in turn, promotes tumour suppressor activity.

Recent attempts to investigate the role of miRNAs in tumour diagnosis have been based on the study of individual targeted miRNAs, or the miRNA expression signature, also termed miRNA expression profile. Researchers have demonstrated that, in addition to the distinction of tumours from normal tissue, miRNA expression was characteristic for tumour type, stage and other clinically relevant variables. Cummins and co-workers

showed overexpression of 18 different miRNAs (tenfold overexpression of six miRNAs) and silencing of 32 miRNAs in colonic tumour compared with normal colonic tissue.

In another study, 28 and 64 miRNAs were differentially expressed in stage I and II colorectal cancer tissue respectively in comparison with paired normal tissue.

Table 1 summarizes the differential expression of microRNAs in colorectal cancer compared with normal flanking tissue identified by various studies. The results of these studies suggest that variability of individual miRNAs with tumour type and stage probably makes the use of a combination of miRNAs a reliable method of detecting cancer status.

Table 1. *Differential expression of microRNAs in colorectal cancer tissues compared with flanking normal tissue as identified in six studies*

Overexpressed miRNAs	Underexpressed miRNAs
miR-17-5p	***miR-145***
miR-21	
miR-29b	
miR-30c	
miR-106a	
miR-107	
miR-191	
miR-221	
miR-223	
miR-19a	***miR-143***
miR-21	miR-145
miR-19a	miR-30c 42
miR-21	miR-133a
miR-29a	miR-145
miR-92	
miR-148a	
miR-200b	
miR-15b 24	
miR-181b	
miR-191	
mirR-200c	

In short, miRNAs more interesting in colorectal cancer are: miR-21, miR-31, miR-34, miR-126, miR-135 miR-143, miR-145, miR-200, miR-342.

- **miR-21** is a strong PTEN suppressor. It's up-regulated in many colorectal tumors. It behaves as an oncogene.
- **miR-31** down-regulates FOXC2 and FOXP3 (pro-apoptotic genes) and is an oncogene.
- **miR-135** suppresses APC (realising beta-catenina). It was found up-regolated in vivo in colorectal carcinoma. It behaves as an oncogene
- **miR-200** targets MLH1 and MSH2 genes and behaves as an oncogene.
- **miR-143** suppresses K-Ras and ERK5 sintesis. It was found down-expressed in colon cancer, *in vivo*. *In vitro*, miR143 inhibition upgrades cell proliferation, so it is behaves as a tumor-suppressor.
- **miR-145** targets IRS1 (insulin-receptor-substrate 1), a well-known mitogen, and therefore it's a tumor-suppressor.
- **miR-126** inhibits AKT. It's down-regolated in many cases of colo-rectal cancer. It's a tumor-suppressor.
- **miR-34** targets p53. A possibile mechanism of p53 loss (beyond his mutation) is the loss or reduction of miR34 for mutilation of gene in CpG islands. (tumor-suppressor).

Chapter 2

2.1 Aims of study

The definition of new molecular prognostic and predictive parameters is one of the most important aims of clinical research in oncology. Ideally, such parameters could be able to better define the prognosis of a single patient and his chance to obtain a clinical response after a specific treatment. The objective of this study is to investigate whether the expression of several micro RNAs are associated with specific clinical outcomes in patients affected by locally advanced rectal cancer treated with neoadjuvant chemoradiation and then after with surgery and adjuvant chemotherapy, and to identify specific signature associated with response to treatment. With the aim to analyze the expression of different miRNA in those patients, prior treatment initiation, rectal biopsies from primary tumor and from normal mucosa will be done for RNA extraction. The Spearman's correlation test, t-test and logistic regression analysis will be used to explore the correlation among these biological factors and the pathological response of the tumors, measured as tumour regression grade (TRG). Biological factors expression was considered as a continuous variable.

2.2 Materials and methods

Patients and treatment

Forty-6 patients with histological diagnosis of rectal adenocarcinoma invading through the intestinal wall or with pelvic lymph node involvement as measured by endorectal ultrasonography (uT3/uT4 or any uT/N+) was treated with neoadjuvant chemo-radiotherapy. Capecitabine was given orally at 825 mg/m2 b.i.d. (two administrations each 12 h apart), from day 1 to 14 every 21 days.

Oxaliplatin was administered i.v. at 50 mg/m2, diluted in 500 ml of glucose solution as a 120-min infusion, at days 1 and 8 of each 21-day cycle.

Pelvic conformal RT was delivered at the daily dose of 1.8 Gy, 5 days a week, up to 45 Gy in 5 weeks.

After surgery, further 4 months of adjuvant chemotherapy with CAP or weekly 5-FU–leucovorin were proposed to all the patients. The oral (CAP) or the i.v. (5-FU–leucovorin) regimen was a patient choice.

Staging procedures had to be carried out within the 2 weeks preceding the treatment start and included blood cell count, biochemistry, blood carcinoembryonic antigen level, thorax–abdomen–pelvis CT scan, total body positron emission tomography (PET), colonoscopy, and endorectal ultrasonography.

At 4–6 weeks after chemoradiotherapy completion, the stage of the disease was re-evaluated by repeating thorax–abdomen–pelvis CT scan, total body PET, and endorectal ultrasonography, in order to assess primary tumour response and to exclude the presence of distant metastases.

Patients were monitored weekly by history, physical examination, and blood count; complete biochemistry was carried out at each cycle. Toxicity was graduated according to National Cancer Institute Common Toxicity Criteria, version 3.0.

The protocol suggested surgery be carried out 6–8 weeks after completion of RT, using the total mesorectal excision technique. However, the choice of the surgical procedure (i.e. abdominoperineal resection or low anterior resection) was at the surgeon's discretion as it was the bridge ileostomy after low anterior resection.

Specimen dissection and mesorectum evaluation were carried out according to the College of American Pathologists protocol for all invasive carcinomas of the colon and rectum (revised version of January 2005; based on AJCC/UICC TNM, 6th edition). Tumour sampling was carried out along the neoplasm major axis; mesorectum was always inked and sampling was carried out in the points of maximum neoplastic infiltration.

Pathologic response was independently scored by two pathologists (FPDA and MRDA) who did not participate in the clinical data gathering, following tumour regression grade (TRG) as described by Mandard. (see Table 1).

Table 1. Tumour regression grade scoring system

TRG 1:	Complete response with absence of residual cancer and fibrosis extending through the wall
TRG 2:	Presence of residual cancer cells scattered through the fibrosis
TRG 3:	Increase in the number of residual cancer cells, with fibrosis predominant
TRG 4:	Residual cancer outgrowing fibrosis
TRG 5:	Absence of regressive changes

Pathological complete response (TRG1) was observed in 9 patients; TRG2 in 19 patients; TRG3 in 12 patients; and TRG4 in 3 patients. Overall, nine patients recurred: five with distant metastases, one with local recurrence, and three with both local recurrence and distant metastases.

Tissue processing

Rectal tumor samples were collected in the operating theater and frozen within 15 min in liquid nitrogen after surgery and stored at -80°C. The collection and use of tumor samples was approved by the local scientific ethical committee and written consent was obtained from the patients.

RNA isolation

Frozen primary tumor tissues (100 mg) were homogenized at 8000 RPM in a *Ultraturrax T 10* at 4°C with 1 ml of Trizol until to obtain a totally liquid phase and total RNA was extracted and purified using *Ambion mirVana RNA isolation kit* (Applied Biosystems, Italy) according to the manufacturer's instructions. RNA was measured by *NanoDrop, ND-1000 Specthophotometer* (NanoDrop Technologies, USA) and quality assessed by *Agilent Bioanalyzer Assay* (Agilent Technologies, Germany), which consists of a *RNA 6000 Nano Kit,* to measure the total RNA integrity and concentration, and a *Small RNA Kit,* to measure the small RNA amount and profile. The RNA kits contain chips and reagents designed for analysis of total RNA and RNA fragments. Each RNA chip contains an interconnected set of microchannels (fig. 1) that is used for separation of nucleid acid fragments based on their size as they are driven through it electrophoretically. The chips are designed only for use with the *Agilent 2100 Bioanalyzer* (Agilent Technologies, Germany).

After their insertion in the Agilent 2100 Bioanalyzer, the chips run and the software shows and save their data. The Agilent 2100 Expert Software automatically provides a RNA Integrity Number (RIN). RIN provides a quantitative value for RNA integrity that facilities the standardization of quality interpretation. Only samples with a RNA quality score (RIN) larger than 5 were further processed and aliquots were stored at -80°C until use.

Total RNA characterization provides information on quality of the total RNA extracted, which may impact the overall RNA quantification and the microarray miRNA profile. The resulting electropherogram typically has at least two distinct peaks representing the 18S and 28S ribosomial RNA (Fig.2).

The small RNA assay characterizes the total RNA sample with an emphasis on the small RNA content of which of a fraction is miRNA. The resulting electropherogram (fig. 3) typically has a number of bands or peaks representing small RNAs ranging in size from 10 to 150 nt. The miRNA portion is represented by the band or peak ranging from 10 to 40 nt. These bands or peaks will vary in abundance depending on the total miRNA preparation. However, the small RNA assay results may not necessarily be an indicator for the miRNA content in the sample, and the miRNA microarray assay is a more sensitive technique for detecting low levels of miRNAs.

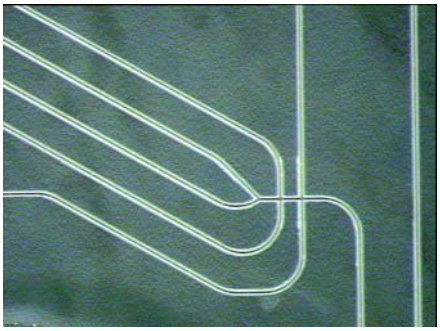
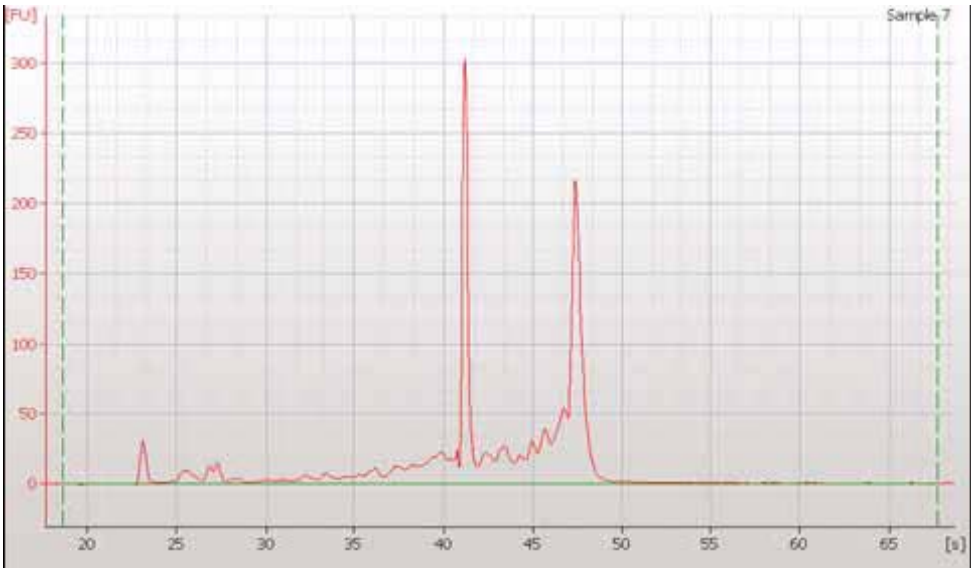
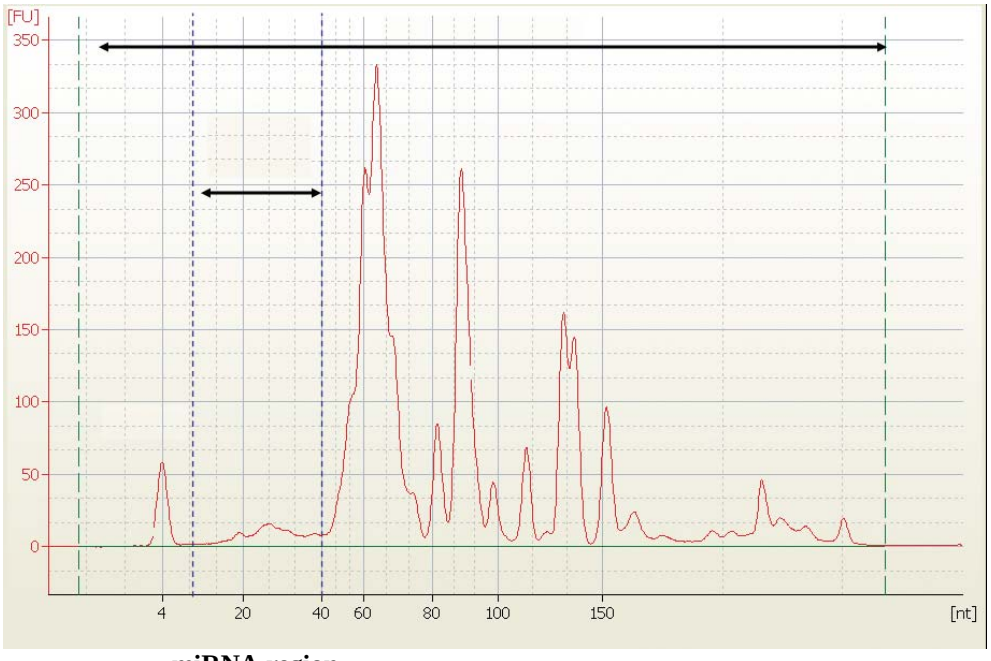


Fig. 1: **Microchannels**



18S 28S

Fig. 2 **RNA peaks of a successful sample run**



miRNA region

Fig.3 **Small RNA Sample Well Results**

miRNAs Microarray Assay

miRNA expression was analyzed using *Agilent miRNA Microarray System* with *miRNA Complete Labeling and Hybriditazion Kit* (Agilent Technologies, Germany), which offers all the necessary reagents for labelling and hybridization on the miRNA microarray.

Agilent's miRNA Complet Labeling and Hyb Kit generates fluorescent miRNA with a sample input of 100 ng of total RNA. This method involves the dephosphorylation of RNA with Calf Intestinal Phosphatase, denaturation by the add of DMSO to total RNA and ligation of one Cyanine 3-pCp molecule to the 3' end of a RNA molecule with greater than 90% efficiency. After labelling reaction, the samples are assembled with the array slide according to the manufacturer's instructions. The arrays are hybridizied for at least 20 hours, in a hybridization rotator that rotates at 20 RPM. The day after, the microarray slides are washed with Gene Expression Wash Buffers. Hybridization signals were detected using the DNA microarray scanner G2505B (Agilent Technologies), and all scanned images were analyzed using Agilent feature extraction software (v9.5.3.1). Data were analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies) and normalized as follows:

(i) values below 0.01 were set to 0.01. (ii) each measurement was divided by the 75th percentile of all measurements to compare one-color expression profile.

2.3 Data analysis

Thirty-nine frozen fresh biopsies of patients affected by a locally advanced rectal cancer treated with neoadjuvant chemo-radiotherapy have been used for this study. Patients have been divided in three groups by TRG:

Group A: patients with TRG 1

Group B: patients with TRG 2-4

Group C: patients not responders

Each group has been equally divided in two subgroups with regard of TRG, with the aim to have a "training set" of 19 patients and a "validation set" of 20 patients.

A microarray platform has been used to determine miRNA expression. Each array is composed by 939 miRNAs, 2689 probes and 15024 spots. First of all, Agilent fluorescence of each array has been measured; then after, background and negative control (which measures not specific fluorescence, for example probe autofluorescence) have been measured by total value of fluorescence of each array (fig.4).

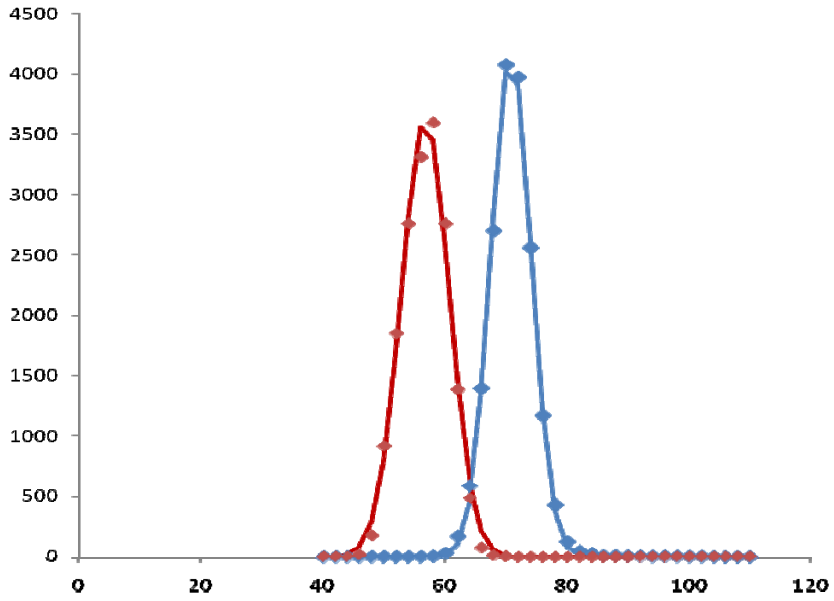


Fig.4: Fluorescence Units (FU) (median pixel)

Reproducibility of fluorescence signals has been tested in replicate spots. Co-relation between duplicates and triplicates has been performed, and co-relation inter and intra-slides has been performed. So, 796 outliers spots (which drift away from media of their probe much more than 2.33 standard deviation and alone increase media of their probe much more than 1.5 times) have been identified and deleted.

Then, not expressed miRNA have been identified as the negative probes in all patients. Thus, 566 of 939 miRNAs (60%) have been identified and deleted. Subsequently, median value of replicated spots of each probe (FU media) have been calculated. Not specific levels (negative control) have been subtracted from FUmedia value and the logarithm in basis of 2 has been calculated. Data have been normalized by linear regression.

p-value of miRNA has been calculated by probability property for independent events. So that, 66 miRNA with p-value < 0.05 have been identified (fig. 5).

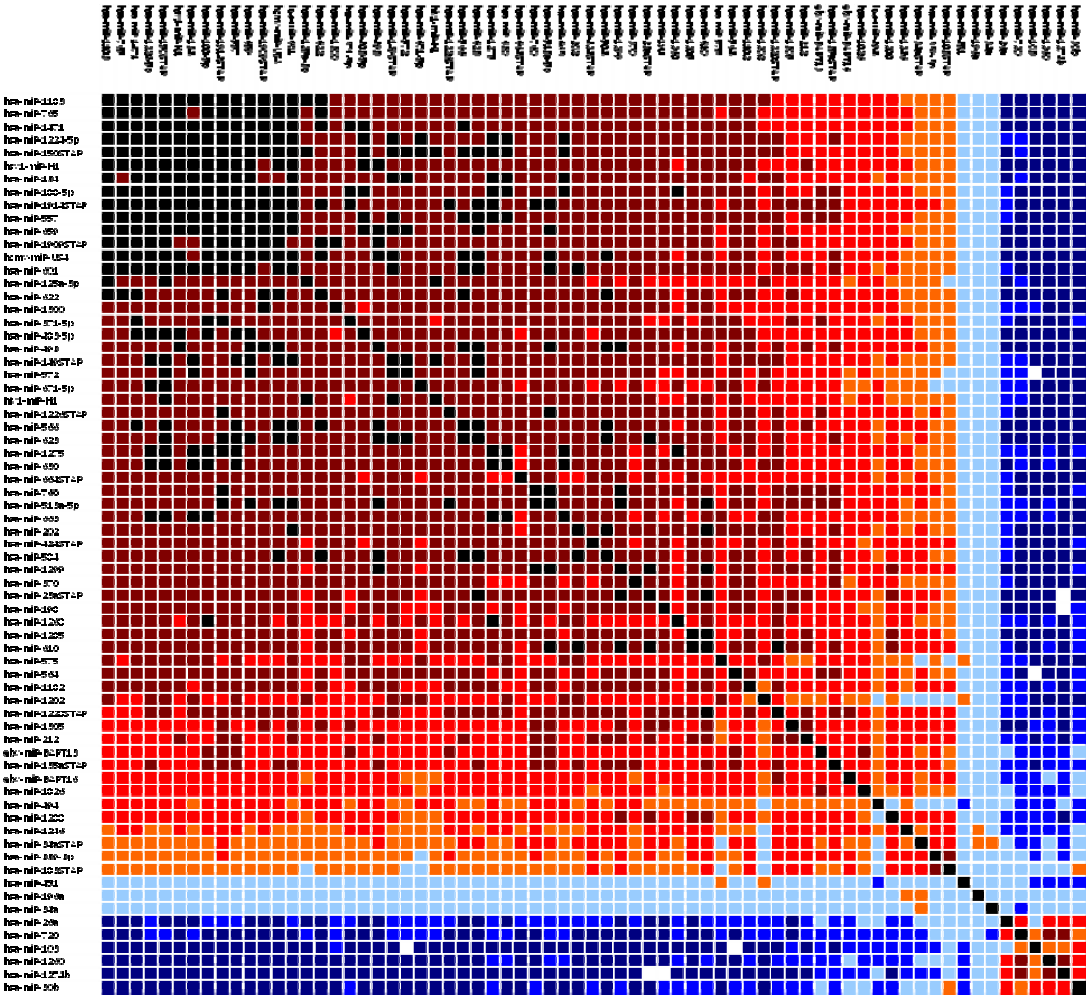
Fig.5: Selection 1 (66 miRNA)

SystematicName	p_{miRNA} A vs BC	FC A/BC	Cluster	Fam	Chr
hsa-miR-765	0.000397	2.88	1	mir-765	1
hsa-miR-1226STAR	0.000418	3.17	1	mir-1226	3
hsa-miR-1183	0.000747	3.02	1	mir-1183	7
hsa-miR-1182	0.000801	3.49	1	mir-1182	1
hsv1-miR-H1	0.000939	3.44	1		
hcmv-miR-US4	0.001057	3.14	1		
hsa-miR-23aSTAR	0.001852	3.14	1	mir-23	19
hsa-miR-1274b	0.001952	0.70	0	mir-1274	19
hsa-miR-483-5p	0.002028	2.43	1		
hsa-miR-622	0.002148	2.97	1	mir-622	13
hsa-miR-125a-3p	0.002157	2.36	1		
hsa-miR-1224-5p	0.002649	2.31	1		
hsa-miR-188-5p	0.002955	2.30	1		
hsa-miR-424STAR	0.003054	2.47	1	mir-322	X
hsa-miR-1471	0.003493	3.12	1	mir-1471	2
hsa-miR-513a-5p	0.004145	2.64	1		
hsa-miR-601	0.004385	2.76	1	mir-601	9
hsa-miR-671-5p	0.004974	2.31	1		
hsa-miR-1285	0.005449	2.27	1		
hsa-miR-1909STAR	0.006209	2.76	1	mir-1909	19
hsa-miR-1299	0.006331	2.77	1	mir-1299	9
hsa-miR-623	0.006572	3.06	1	mir-623	13
hsa-miR-659	0.006688	3.15	1		22
hsa-miR-630	0.007126	2.46	1	mir-630	15
hsa-miR-202	0.007961	2.93	1	mir-202	10
hsa-miR-1914STAR	0.008357	1.89	1	mir-1914	20
hsa-miR-1228STAR	0.008409	2.44	1	mir-1228	12
hsa-miR-1300	0.009179	2.05	1		
hsa-miR-30b	0.009904	0.69	0	mir-30	8
hsa-miR-149STAR	0.01032	3.18	1	mir-149	2
hsa-miR-134	0.010548	2.15	1	mir-134	14
hsa-miR-150STAR	0.010692	2.01	1	mir-150	19
hsa-miR-493	0.011046	2.59	1	mir-493	14
hsa-miR-564	0.011733	2.06	1	mir-564	3

SystematicName	p_{miRNA} A vs BC	FC A/BC	Cluster	Fam	Chr
hsa-miR-557	0.01176	2.59	1	mir-557	1
hsa-miR-663	0.012174	2.09	1	mir-663	20
hsa-miR-720	0.013643	0.66	0	mir-720	3
hsa-miR-575	0.0137	2.35	1	mir-575	4
hsa-miR-610	0.013852	2.86	1	mir-610	11
hsa-miR-1275	0.014581	1.73	1	mir-1275	6
hsa-miR-371-5p	0.015369	2.28	1		
hsa-miR-1826	0.015649	1.73	0		16
hsa-miR-183STAR	0.018183	1.56	0	mir-183	7
hsa-miR-370	0.018404	2.48	1	mir-370	14
ebv-miR-BART16	0.018733	2.11	0		
hsa-miR-1246	0.019089	2.39	0	mir-1246	2
hiv1-miR-H1	0.019205	2.04	1		
hsa-miR-760	0.01971	2.67	1	mir-760	1
hsa-miR-664STAR	0.022419	1.83	1	mir-664	1
hsa-miR-1305	0.023204	1.80	1	mir-1305	4
hsa-miR-26a	0.023411	0.75	0		
hsa-miR-198	0.02588	2.35	1	mir-198	3
hsa-miR-1268	0.030025	1.84	1	mir-1268	15
hsa-miR-103	0.031032	0.80	0		
hsa-miR-1260	0.031227	0.63	0	mir-1260	14
hsa-miR-135aSTAR	0.031383	2.80	1		
hsa-miR-584	0.032723	2.34	1	mir-584	5
hsa-miR-212	0.034728	1.74	1	mir-132	17
hsa-miR-494	0.035024	1.71	0	mir-154	14
hsa-miR-1288	0.036946	2.05	0	mir-1288	17
hsa-miR-339-3p	0.038988	1.65	0		
ebv-miR-BART13	0.041695	1.80	1		
hsa-miR-1202	0.042337	1.84	1	mir-1202	6
hsa-miR-34aSTAR	0.04236	1.78	0	mir-34	1
hsa-miR-566	0.044094	2.53	1	mir-566	3
hsa-miR-572	0.049083	2.68	1	mir-572	4

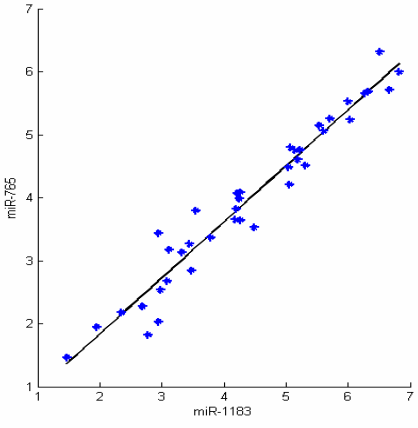
Fluorescence levels of 66 selected miRNA have been correlated in all patients.

Correlation Matrix between miRNA



R di Pearson

Correlation example between 2 miRNA



With the aim to obtain a miRNA list, which discriminates Group A patients, to validate in RT-PCR, to 66 miRNA list have been applied further filters calculated by 4 criteria obtained with Microsoft Excel and 2 criteria with the script of "R", with or without Background subtraction:

Criterion 1: Probe Validation p <0.1 A s BC

Criterion 2: miRNA Validation p< 0.15 A vs BC

Criterion 3: All Dataset miRNA pvalue < 0.05 (used to obtain first selection of miRNA)

Criterion 4: All Dataset miRNA qvalue <0.15

Criterion 5: R without subtraction of BG q value <0.1

Criterion 6: R with subtraction of BG q value <0.1

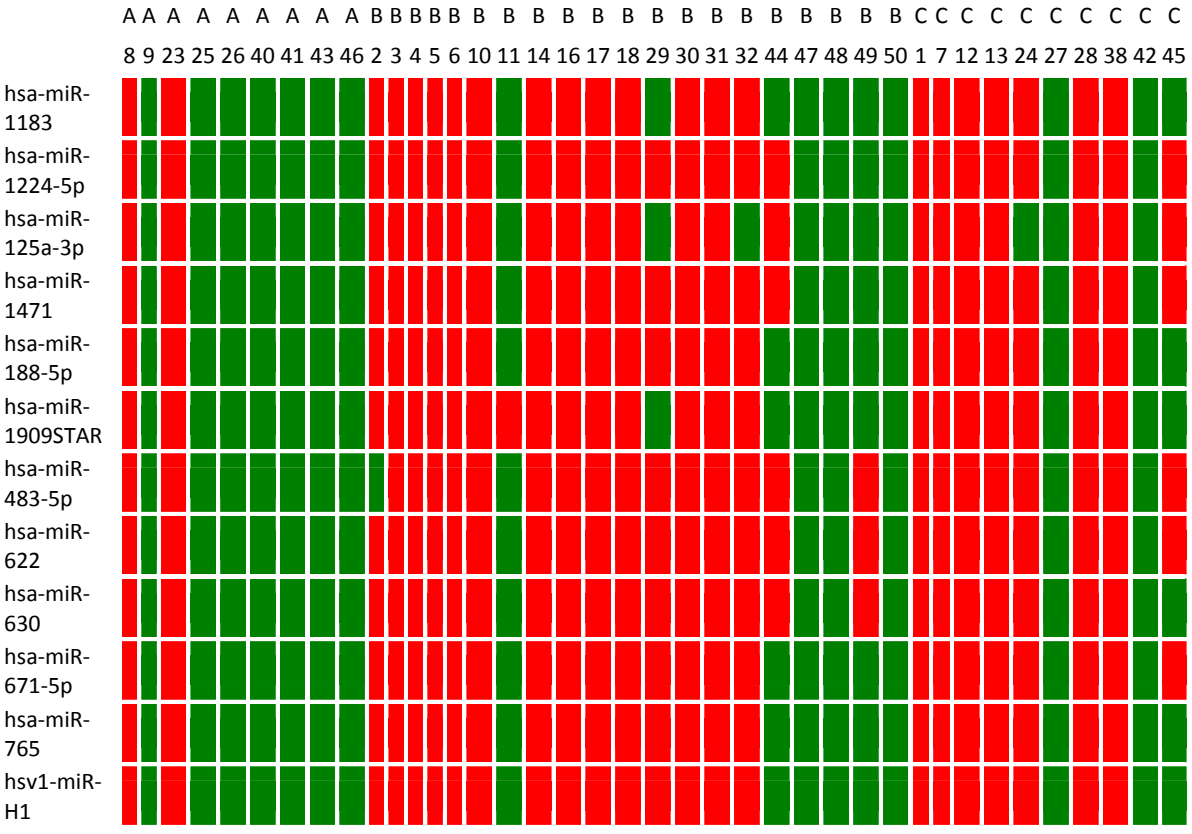
2.4 Results

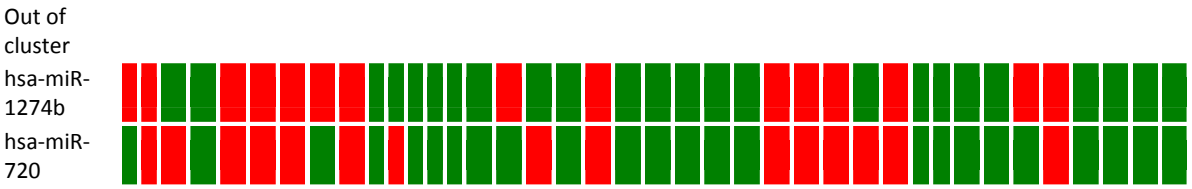
We have been able to identify 14 miRNA which are specific for Group A (TRG 1) with respect of other groups (B,C). These miRNAs have fluorescence levels higher in Group A than B and C; moreover, they have a fluorescence value which changes in the same way in patients of these group, namely they are strongly linked each other and form a "cluster". This cluster correlate with TRG1 with a specificity of 83% and sensitivity of 78%. The cluster may be an interesting marker: all miRNA in the cluster are overexpressed together in the same patients of group A (fig.6).

Fig. 6: List of 14 selected miRNA specific for Group A

SystematicName	R p A vs BC	R p B vs C	R p AB vs C	Amedia	FC A/BC	FC AB/C	Cluster	Fam	accession	Chr	Chr coord	Chr coord
hsa-miR-765	0.0004	0.6798	0.5186	5.1333	2.8786	1.6013	1	mir-765	MI00051161	15690592	15690603	6
hsa-miR-1183	0.0007	0.7948	0.4515	5.6190	3.0236	1.5772	1	mir-1183	MI00062767	21510676	21510764	
hsv1-miR-H1	0.0009	0.5762	0.8552	5.1133	3.4400	1.3587	1					
hsa-miR-1274b	0.0020	0.2307	0.2613	10.7036	0.6977	0.7538	0	mir-1274	MI000642719	58024375	58024441	
hsa-miR-483-5p	0.0020	0.7997	0.4308	6.6362	2.4262	1.4091	1					
hsa-miR-622	0.0021	0.7406	0.6013	5.3691	2.9724	1.6474	1	mir-622	MI000363613	90883436	90883531	
hsa-miR-125a-3p	0.0022	0.8538	0.6941	8.1461	2.3553	1.4111	1					
hsa-miR-1224-5p	0.0026	0.9432	0.5387	6.2904	2.3117	1.2981	1					
hsa-miR-188-5p	0.0030	0.9048	0.5491	6.1835	2.2978	1.2825	1					
hsa-miR-1471	0.0035	0.8154	0.5703	5.0645	3.1198	1.4947	1	mir-1471	MI00070762	23275695	23275700	8
hsa-miR-671-5p	0.0050	0.7951	0.5686	6.0724	2.3097	1.1934	1					
hsa-miR-1909STAR	0.0062	0.6870	0.7419	5.0301	2.7638	1.2836	1	mir-1909	MI000833019	1816158	1816237	
hsa-miR-630	0.0071	0.3857	0.8753	6.0162	2.4632	1.0758	1	mir-630	MI000364415	72879558	72879654	
hsa-miR-720	0.0136	0.3093	0.3082	10.3321	0.6612	0.7368	0	mir-720	MI00066543	16405912	16405923	8

This miRNA profile may be proposed as a specific "signature" to validate in RT-PCR.





Validation of the microarray results by real-time qPCR

To detect the expression level of miRNA by real-time qPCR, TaqMan® microRNA assay (Applied Biosystems) was used to quantify the relative expression levels of 14 miRNA identified (see fig. 5). cDNA was synthesized by Taqman miRNA RT Kit (Applied Biosystems). Total RNA (10 ng) in 5 ul of nuclease free water was added to 3 ul of 5× RT primer, 10× 1.5 ul of reverse transcriptase buffer, 0.15 ul of 100 mM dNTP, 0.19 ul of RNase inhibitor, 4.16 ul of nuclease free water, and 50 U of reverse transcriptase in a total volume of 15 ul. The reaction was performed for 30 min at 16°C, 30 min at 42°C, and five min at 85°C. All RT reactions were run in triplicate. Chromo 4 detector (BIO-RAD, Hercules, CA, USA) was used to detect miRNA expression.

2.5 Discussion

MicroRNAs are small RNAs that regulate gene expression at the post-transcriptional level. miRNAs exert an important role in the regulation of biological processes, and abnormal expression of miRNAs are associated with different cancers. Considering the strong association between genetic

alterations and neoplastic diseases, it is not surprising that there is a special focus on the correlation between miRNAs and cancer. Several experimental studies on colorectal cancer, the most common cancer site and furthermore the second most common cause of death due to cancer, have highlighted the critical role of miRNA in regulation of wellknown oncogenic and tumour suppressor signalling pathways.

Furthermore, several investigations have described the ability of microRNA expression patterns to predict prognosis in colon cancer and support diagnosis of poorly differentiated tumours. Currently, two different approaches are applied to investigate the association between miRNA and colorectal cancer (CRC). On the one hand, miRNAs seem to regulate many known oncogenic and tumour suppressor pathways involved in the pathogenesis of CRC. This is of particular interest in colorectal neoplasms as many proteins involved in key signalling pathways in this tumour, like p53, RAS and epithelial–mesenchymal transition (EMT) transcription factors as well as members of the PI-3-K and the Wnt/β-catenin pathway seem to be affected by miRNA regulation. Their dissection in functional studies is critical for a better understanding of cancer biology, eventually aiming for the identification of novel pharmaceutical targets. On the other hand, expression profiles of hundreds of different miRNAs have been shown to bear a much higher potential as biomarkers than their mRNA

counterparts. This allows a prediction of prognosis and a distinction of certain disease entities including colorectal cancer sub-types.

In order to investigate miRNA differential expression in human colorectal cancer, we analyzed by microarray and real-time PCR the expression of 939 mature miRNAs in total RNA extracted from 39 rectal fresh frozen biopsies, with the aim to found a “signature” that correlates with the response to treatments.

Total RNA was extracted from biopsies of patients affected by a locally advanced rectal cancer, treated with neo-adjuant chemo-radiotherapy, follwing by surgery. RNA concentration was tested by Nanodrop ND 1000 spectrophotometer (fig 7) and by Agilent Bioanalyzer Assay to measure the total RNA integrity (RIN) and concentration (fig. 8). Also small RNA was tested by Small RNA Kit to characterize the fraction of miRNAs (fig. 9).

The expression level of mature miRNAs found their biopsy specimen, obtained prior to the combination therapy, were quantified using microarray analysis (fig. 10). The miRNA expression pattern was classified based on the final pathological response to the combination therapy.

We found that the expression level of 14 miRNAs were significantly different in patients with TRG 1 if compared with patients with other TRG, with a sensitivity of 78% and a specificity of 80%. The miRNA expression pattern is associated with therapeutic outcome and may be a specific

"signature". This information could be utilized as a novel biomarker to predict drug response.

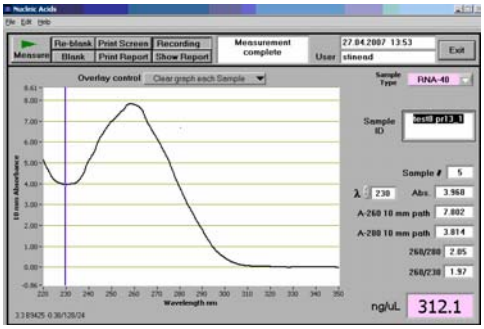


Fig. 7: **Nanodrop Spectrophotometer evaluation**

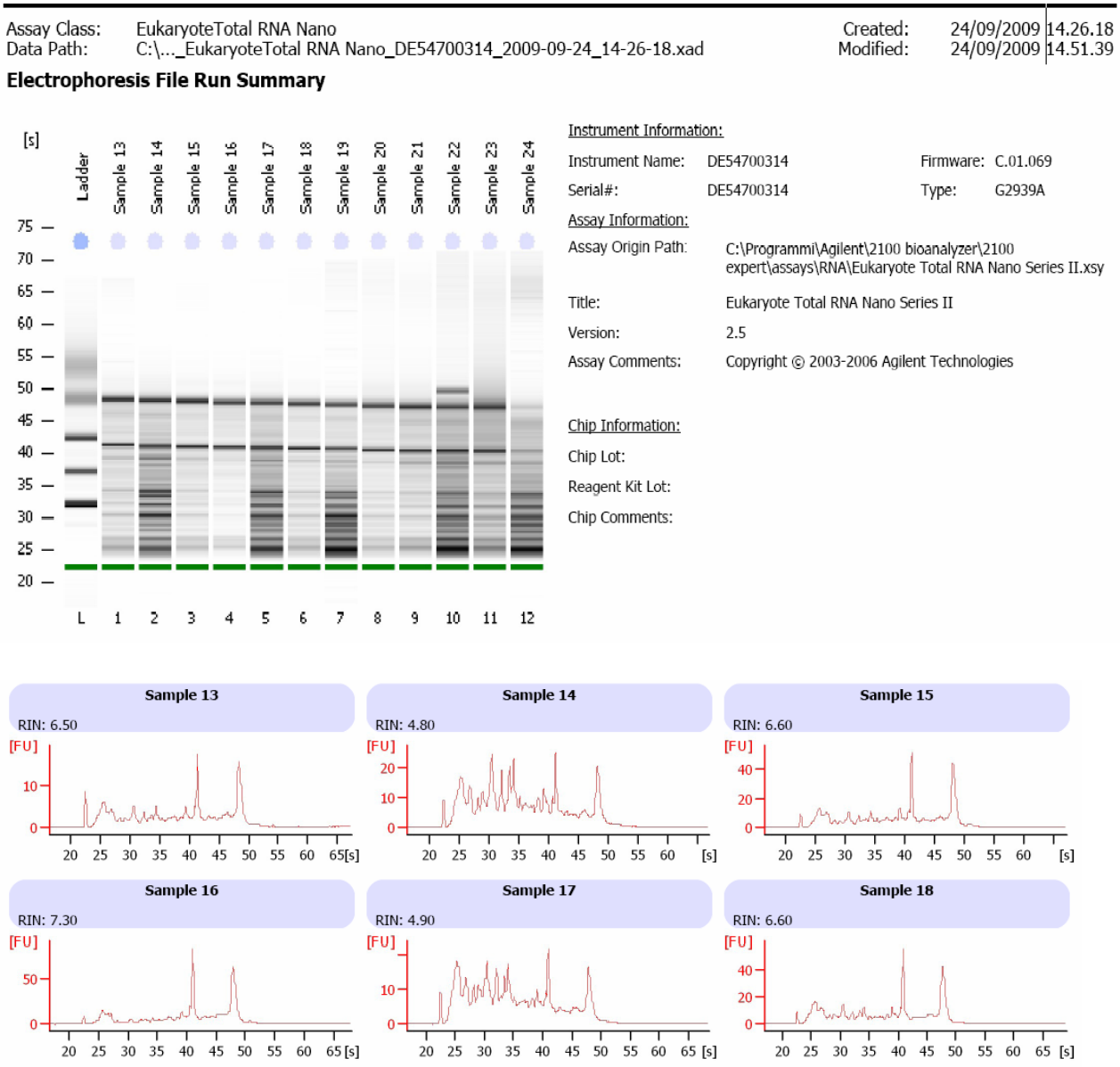


Fig. 8: Total RNA Integrity Number (RIN)

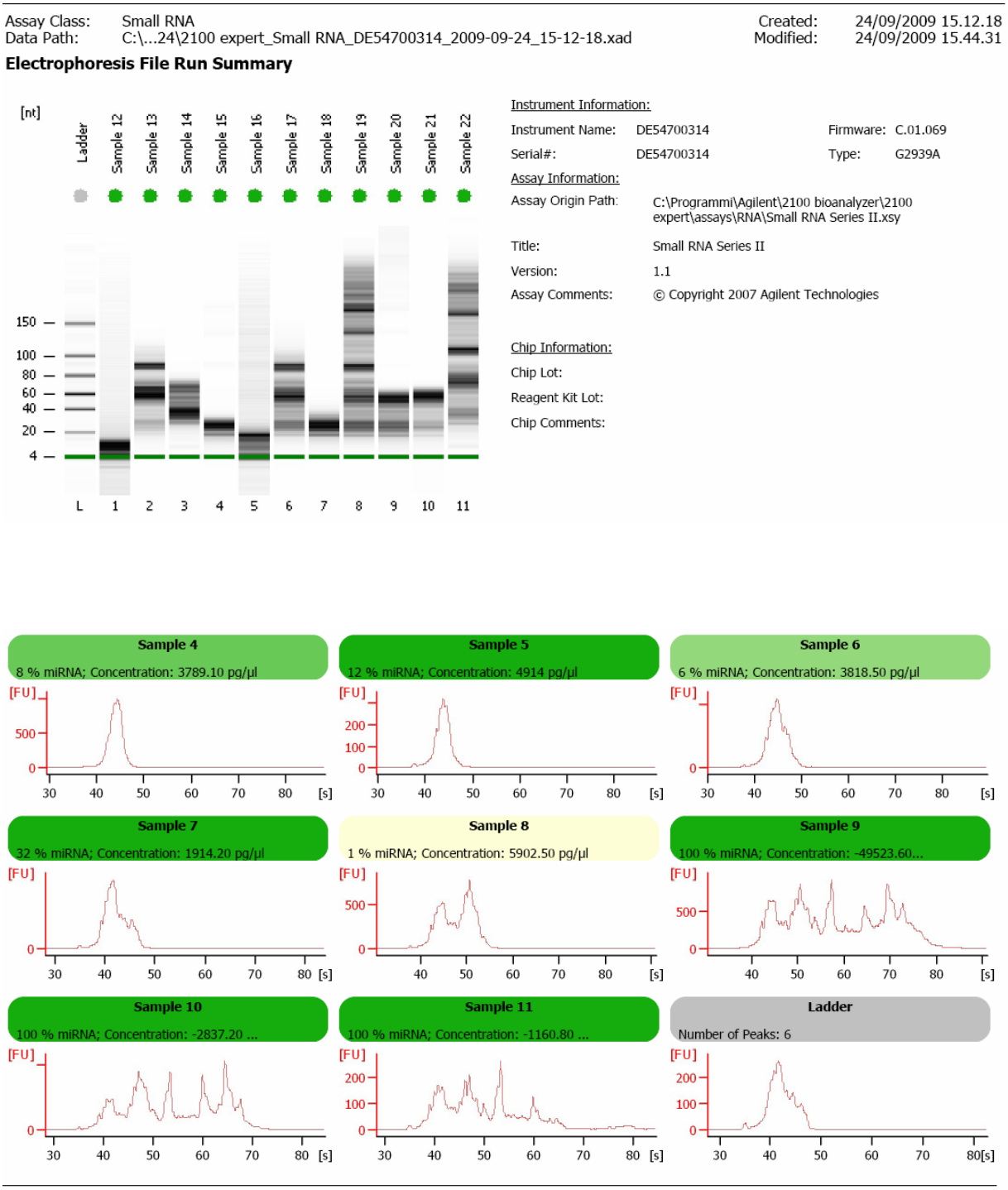
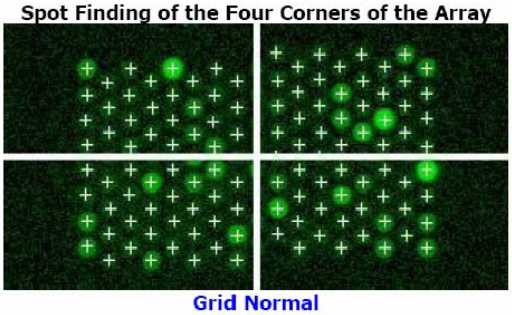


Fig. 9: Small RNA (miRNAs fraction 40-50 nt)

QC Report - Agilent Technologies : miRNA

Date	Thursday, January 14, 2010 - 12:06	Grid	021827_D_F_20090519
Image	US45102933_252182711901_S01_H [1_1]	BG Method	No Background
Protocol	miRNA_105_Jan09 (Read Only)	Background Detrend	On(FeatNCRange, LoPass)
User Name	Administrator	Multiplicative Detrend	False
FE Version	10.5.1.1	Additive Error	2
Sample(red/green)		Saturation Value	1213068

Spot Finding of the Four Corners of the Array



Grid Normal

	Feature	Local Background
Non Uniform	0	5
Population	981	1

Net Signal Statistics

Non-Control probes:

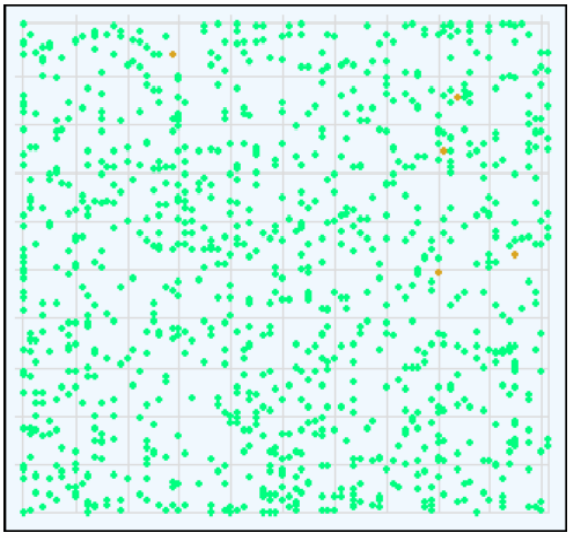
# Saturated Features	0
99% of Sig. Distrib.	3520
50% of Sig. Distrib.	32
1% of Sig. Distrib.	24

Negative Control Stats

Average Net Signals	35.21
StdDev Net Signals	2.12
Average BG Sub Signal	3.56
StdDev BG Sub Signal	2.09

Spatial Distribution of All Outliers on the Array

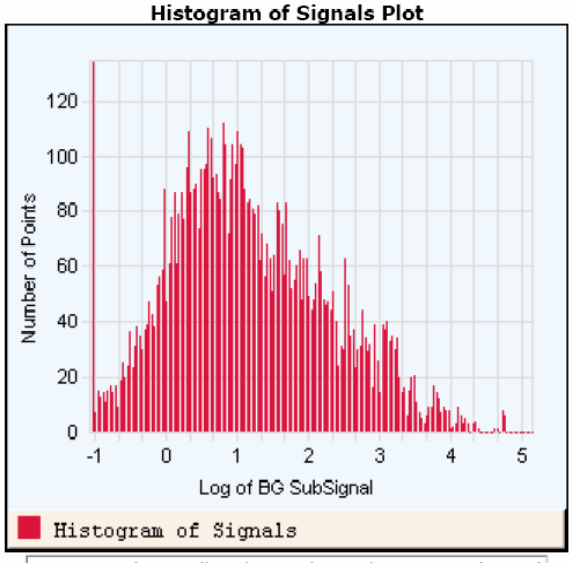
192 rows x 82 columns



FeatureNonUnif = 0(0.00%)

Feature Population Outliers = 981(6.23%)

Histogram of Signals Plot



Features (NonCtrl) with BGSubSignal < 0: 7629 (Green)

BG NonUniform BG Population

Green FeaturePopulation Green Feature NonUniform

Fig. 10

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