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Expression profile of miRNA in colorectal adenocarcinoma

DOCTORAL THESIS - SUMMARY

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Introduction

Colorectal cancer represents a multifactorial/polygenic disease, the result of the successive accumulation of genetic and epigenetic abnormalities that sequentially activate oncogenes or inactivate tumor suppressor genes.

From the moment of initial diagnosis till the point a complete clinical response is obtained, the histopathological examination plays a key role in the care of colorectal cancer patients. In this regard, the anatomopathological staging of the large bowel's neoplasms is recognized as the most accurate and constant tool for evolution and planning of therapy. However, clinicopathological parameters are not always sufficient, because morphologically, similar tumors may be different in terms of molecular architecture. In addition, analysis of molecular markers has been shown to have prognostic significance, regardless of the stage, thus contributing to better stratification of the colon and rectal adenocarcinomas. Consequently, the main objective of the paper includes the identification of new efficient diagnostic methods and the exploration of the molecular mechanisms underlying the development and progression of CRC.

Therefore, as our knowledge of the eukaryotic cell genome's structure and organization evolved, non-coding RNAs appeared as new molecular targets. Ribonucleic acid genes are represented by two types of RNA molecules, one class plays an active role in cellular infrastructure and protein synthesis (messager RNA), whereas the other class entails functional molecules with versatile and vital roles in cellular homeostasis, namely, non-coding RNA (ncRNA) (1).

Micro-ribonucleic acids (miRNAs) refer to short nuclear RNA sequences present in prokaryotes and eukaryotes cells with important functional roles in a wide range of physiological and pathological processes. Since the number of miRNA genes known is significantly lower than that of the protein-coding genes they modulate, an exploration of the miRNA profiles in adenocarcinomas of colon and rectum provides a detailed view of the molecular structure (2).

It is now widely accepted that the altered functionality of miRNAs plays an important role in various biological and cancer-related processes, such as control of cellular homeostasis, differentiation, cell growth, and apoptosis. In colorectal tumorigenesis, many researchers have used the specific expression patterns of human miRNAs to understand the involvement of these

regulatory molecules in the diagnosis and prognosis of this type of cancer.[6] Due to high tissue specificity, altered stability, and unique expression in tumor development, some of these deregulated mature miRNAs might help distinguish colorectal cancer from other colon-related diseases, thereby representing a new field of molecular diagnosis and prognosis of CRC.

A complete understanding of miRNAs role in colorectal carcinogenesis is far from being fully understood because their expression can oscillate and can be influenced by not only the unique genetic and epigenetic characteristics of different populations, but also by the environmental factors associated with each region. In this context, the expression of miRNAs in Romania has been studied very superficially. In order to full this research gap, the current study will complete the current histopathological and paraclinical diagnosis before being presented to the clinician in an integrated model.

In my doctoral research, I aimed to investigate and compare the expression of a panel of mature miRNA genes in colorectal adenocarcinoma (CRC) and normal adjacent non-tumoral tissue (NATS), as a result of the data collected from patients in south-east Romania, and to further explore their association with clinicopathological features. The ability of these selected miRNAs to function as potential biomarkers, thus discriminating between CRC and NATS and, their potential as indicators in CRC prognosis was also examined.

In light of these data, miRNA genes have relevant biological and biomedical consequences in the detection and evolution of cancer, with their inadequate expression denoting an almost universal feature in human malignancies.

The general part

The current stage of research

The first part of the doctoral thesis follows the current state of knowledge in the field of chosen research and is structured in 3 chapters that address theoretical notions about colorectal cancer-general considerations, the molecular substrate of colorectal carcinogenesis, and microribonucleic acid genes. The second part presents the research hypotheses, main objectives, materials, and methods used, as well as the original results obtained in the doctoral study.

The chapter entitled "Colorectal cancer-general considerations" presents comprehensive information on the epidemiology, etiology, pathogenesis, diagnosis, and staging of colorectal cancer. The data presented show the epidemiological situation of colorectal cancer (CRC) worldwide, a condition that continues to be in continuous dynamic expansion, with approximately 1.3 million new cases diagnosed and 694,000 recorded deaths in 2012, thus representing 10% of all human neoplasms. (http://gco.iarc.fr) (3). In Romania, CRC ranks second with 8,660 after lung cancer, in terms of the number of cases, and terms of distribution by sex, both men and women, RCC ranks third with 5,890 new cases in men (after lung and prostate cancer) and 3,310 new cases in women (after breast and cervical cancer) (4).

Although life expectancy has grooved considerably in recent years as a result of improved clinical management and diversification of treatment options, CRC continues to be the leading cause of death among gastrointestinal tumors. Because this neoplastic condition is in generally asymptomatic in the early stages (stage I - II) and becomes symptomatic in the late stages (stage III-IV), when the tumor has already spread to adjacent tissues and/or loco-regional lymph nodes, it is imperative precise anatomopathological staging and early detection of premalignant lesions, steps that are essential in the management of patients with CRC (5).

Several histological variants of CRC tumors are listed in the WHO classification. The most common are those with epithelial origins (carcinomas), such as adenocarcinoma, "Signer-ring" carcinomas, medullary carcinomas, serrated carcinoma, or comedo-cribriform carcinomas (6). This study focuses on the most common subtype of carcinoma, adenocarcinoma, and therefore the term “colorectal cancer” or “CRC” will be used to designate this subtype.

Colon or rectum neoplasm is defined as a carcinoma, namely adenocarcinoma, which usually develops from a neoplastic precursor, such as adenomas, hyperplastic, mesenchymal, or inflammatory polyps that may be tubular, villous, tubule-villous, or serrated-sessile in structure (7).

Histopathological staging of CRC is a key factor in the planning therapy and predicts the evolution of the disease and it is based on the use of a universally accepted system to describe the local extension of the primary tumor (T), its dissemination to locoregional lymph nodes (N) and extension to other organs - metastasis (M) namely, the Tumor-Node-Metastasis (TNM) system proposed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (8).

The chapter entitled ***"Molecular substrate of colorectal carcinogenesis"*** describes the genomic, epigenomic, and transcriptomic heterogeneity of colorectal carcinogenesis. The interdependent relationship between molecular changes and tumor progression postulated for the first time by Vogelstein and Fearon in 1990 represented a model of appearances and evolution of CRC for more than two decades, this paradigm of genetic and epigenetic events in colorectal cancer, constituting an example of pathophysiological evolution for many other cancers (9).

Three molecular mechanisms are involved in CRC, namely, chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylation phenotype (CIMP).

Chromosomal instability, also known as the adenoma-carcinoma sequence, is the most common type of genetic imbalance, found in 85% of CRC cases. The underlying mechanisms of CIN include the loss or gain of chromosomal segments or entire chromosomes at an increased rate compared to normal cells, telomeres dysfunction as a response to DNA damage, and changes of genes expression involved in cellular homeostasis (10).

The functional disorder of the post-replicative system (MMR), responsible for repairing the wrong pairings between complementary base pairs, is often the target of external or internal mutagens. In humans, the MMR system involves the intervention of a system composed of 6 mutator enzymes, which recognize replication errors, encoded by the genes hMSH2, hMSH3, hMSH6, hMLH1, hPMS1, and hPMS2. This phenomenon occurs frequently in the case of microsatellites, which are very short repeats of 1-6 bp, mono-, bi-, tri- or tetranucleotides of type (CA)n or (A)n, uniformly distributed in the genome, through which a microsatellite germinal

allele presents the addition or deletion of short repetitive units, which changes its somatic length, named microsatellites instability – MSI (11).

Also, recent research on CRC has revealed the existence of loci that are methylated in the group, the phenomenon called CpG island methylator phenotype (CIMP), observed in over 15% of cases. Depending on the degree of hypermethylation, CRC can be divided into the methylator phenotype of high-grade CpG islands (CIMP-H) and methylation phenotype of low-grade CpG islands (CIMP-L) (12).

The chapter namely ***"Micro-ribonucleic acid genes"*** presents information on the discovery, biogenesis, mechanism, and role of microRNA genes in human neoplasms.

Functionally, microRNAs (miRNAs) are a new class of small, highly phylogenetically conserved non-coding RNAs molecules with lengths between 18-25 nt, with a role in the management of gene expression at the post-transcriptional level (13). Typically, miRNA genes are initially transcribed into the nucleus as longer primary transcripts guided by RNA polymerase II (pri-miRNAs), which are subsequently enzymatically cleaved by the Drosha into small miRNAs precursor (pre-miRNA). These pre-miRNAs are comprised of 70 nucleotides with hairpin stem-loop structures and are translocated into the cytoplasm through the assistance of Exportin-5 to undergo final maturation, within a functional miRNA to approximately 22 nucleotides catalyzed via RNase III endonuclease Dicer (14)

Specifically, miRNAs exert their functionality in post-transcriptional modulation of gene expression through direct binding to the 3' untranslated region (UTR) of specific messenger RNA targets (mRNAs), thereby leading to cleavage and degradation or suppression of translation (15). The latest version of the miRBase database contains ~ 1.917 entries of mature human miRNAs (http://www.mirbase.org), which can be classified into clusters and families based on seed sequence or genomic relatedness, able to regulate the expression of one-third of human protein-coding genes (16).

Predominantly, all pathophysiological processes appear to be at least partially regulated by miRNA molecules (17).

Special part

Personal contribution

Research hypotheses

Analyzing the previously published data and consulting the Sanger Center Registry databases (www.sanger.ac.uk/Software/Rfam/miRNA/index) and miRBase (www.mirbase.org) in the doctoral study, I set out to test the following ***research hypotheses***:

1. If the differentiated expression of selected miRNA genes is associated with different stages of colorectal cancer pathogenesis, because it has been shown that miRNAs can have a prognostic significance, independent of stage, thus contributing to better stratification of the colon and rectal adenocarcinomas.
2. Whether miRNA molecules can serve as potential tissue biomarkers with high specificity and sensitivity among patients with colorectal cancer in Romania, given that their expression can oscillate and be influenced by the unique genetic and epigenetic characteristics of different populations, as well as by socio-economic factors associated with each region.

The main and secondary objectives of the thesis resulted from the hypotheses formulated above.

Main objectives:

1. Analysis of demographic, morphophonological, and immunohistochemical variables of the cases included in the study.
2. Quantification of selected miRNA and mRNA gene expression from tumor and normal adjacent non-tumoral samples using the RT-qPCR method.
3. Evaluation of the clinical, diagnostic, and prognostic utility of selected miRNAs as potential tissue biomarkers.
4. Evidencing a possible correlation between the expression of selected miRNAs, respectively mRNAs with clinical and histopathological variables.
5. Establishing the association between miRNA and mRNA genes with the identification of possible miRNA-mRNA duplexes in CRC.

Secondary objectives:

1. Processing of surgical specimens and biopsies for histopathological and immunohistochemical diagnosis.
2. Isolation and purification of tRNAs molecules, including miRNA species from tumor tissue and paired adjacent non-tumor tissue samples (NOR).
3. Determination of purity, quantity, and integrity of RNA samples through spectrophotometry, fluorometry, and microcapillary electrophoresis.
4. Reverse-transcription of RNA molecules from tumor samples, respectively NOR in complementary DNA (cDNA), stage performed with stem-loop specific primers for miRNA species and with hexameric primers with random sequence (random primers) in the case of mRNA molecules.
5. Amplification and quantification of the mature miRNA gene expression (hsa-miR-30c, -375, -195, -214, -144, -145, -143, -299, -21, -141, -182, -183 and -370) and mRNA (PTEN, TP53, MSH6, mTOR and PIK3CA) through the qPCR technique using TaqMan®MGB specific hydrolysis probes.
6. Normalization, analysis, and interpretation of data obtained using statistical software.

Research methodology

Case selection

Tumor samples with paired adjacent normal tissues (harvested at > 5 cm from the cancerous tissue) were collected from 41 patients diagnosed with CRC at the Clinical Emergency County Hospital „Sf Apostol Andrei" in Constanta, Romania. The Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (No: 30367/23.06.2020) approved the study and all eligible patients provided written informed consent. Immediately after the surgical resection, a section of each sample (tumor and non-tumor) was stained with hematoxylin and eosin and was evaluated by an experienced pathologist. All tumor specimens used in this study were histologically classified as colon or rectum adenocarcinoma. The histological tumor stage and differentiation grade was classified using American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system, in accordance with the standards set by the World Health Organization (WHO) (8).

Immediately after the surgical resection, CRC tissues and NATS (located at least 5 cm from the tumor site) were stabilized in RNAlatter® solution and frozen at -80 °C until required for further processing. Subsequently, clinicopathological features of patients' characteristics: age, sex, tumor location, TNM stage, tumor differentiation degree, and eventual metastasis were obtained from observation sheets and pathology reports.

Research Methods

MiRNA/mRNA extraction and quantification

Total RNA including miRNA molecules was isolated from the tissue samples using a miRNeasy kit (Qiagen, Germany) closely following the manufacturer's recommendations. We started with 30 mg of tissue which was thoroughly homogenized in 750 µl QIAzol Lysis Reagent for 90 s. Thereafter, 140 µl of chloroform was added to tissue homogenate and after 5 min incubation at RT, the sample was centrifuged for 15 min at 12.000 rpm at 4 °C. The upper aqueous phase containing RNA was transferred and precipitated in a new Eppendorf tube by adding 1.5 volumes of 100% ethanol. Approximately 650 µl of the precipitated sample was transferred to a RNeasy® Mini column placed in an appropriate collection tube and centrifuged at 12.000 rpm for 1 min at RT. After centrifugation, the filtrate was discarded and 700 µl wash buffer RW1 was pipetted and centrifuged at 12.000 rpm for 1 min. Next, 500 µl of wash buffer RPE was pipetted and centrifuged at 12.000 rpm for 1 min at RT. To dry the membrane, the column was centrifuged at maximum speed for 1 min. Finally, the column was placed in a new tapered collection tube, and 30 µL RNase-free water was added and centrifuged at maximum speed for 1 min to collect an eluate.

The purity and yield of the RNA solutions were assessed by measuring the optical density at 260/280 nm using a NanoDrop One™ Spectrophotometer (Thermo Fisher Scientific, USA), where a ratio A260/A280 = 2 – 2.1, and A260/A230 > 2 was considered acceptable. The concentration of the samples was measured with Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, USA) using the Qubit RNA HR (High-Range) Assay Kit. Furthermore, the RNA integrity number (RIN) was established using the 2200 TapeStation Bioanalyzer (Agilent Technologies GmbH, Germany) with an RNA HS ScreenTape kit.

Reverse transcription of miRNA to complementary cDNA and qPCR reaction

Selected human miRNAs were reverse-transcribed to complementary DNA (cDNA) using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, San Diego, CA). Each reaction was initiated using an RNA-specific stem-loop for reverse transcription (RT) (Table 1). The RNA concentration was set between 1 and 10 ng per 15 µl of RT reaction. Each 15 µl RT reaction consist of 7 µl master mix (0.15 µl dNTP mix, 1 µl Multiscribe RT enzyme, 1.5 µl 10 x RT Buffer, 0.19 µl RNase inhibitor and 4.16 µl Nuclease-free water), 3 µl primer, and 5 µl RNA sample. Samples were incubated in a thermocycler with the following sequence: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then cooled to 4°C.

The complementary DNA strand for selected targets of miRNAs was synthesized using a specific sequence TaqMan®MGB Assay. For the 20 µl reaction mix, 10 µl of TaqMan® 2 × Universal PCR Master Mix was added to 1.33µl of the product from the RT reaction, 7.67µl of RNase-free dH_2O, and 1µl of TaqMan® Small RNA assay (20X). The quantitative real-time polymerase chain reaction analysis (qPCR) was performed in triplicate for each sample using the ABI 7500 Fast qPCR instrument for 40 cycles, where each cycle contained a denaturation step at 95°C for 3 s, and an annealing step at 60°C for 30 s, followed by the extension of the primers with cleavage of the probe. Fluorescence was detected at the end of each cycle. A negative control without a template was used with all the qRT-PCR runs.

Normalization and quantification of qPCR data

In order to estimate the expression levels of the selected genes in this research, relative quantification was used due to its superior flexibility and inherently low variation in sample preparation. The method involves comparing the Ct values (threshold cycle was used as measurement unit) obtained for the gene of interest in the tumor tissue with a calibrator, in this case, a sample from the adjacent normal tissue. In these terms, Ct values in tumor and non-tumor samples are normalized to a reference gene in the same sample, a gene whose expression does not change in tested conditions. In the present study, two controls were used to normalize gene expression for miRNA, respectively miR-26b and miR-92N ($P = 0.60$, $P = 0.58$; paired test) and GADPH ($P = 0.29$) for mRNA species. For each sample, the reactions were performed in triplicate, for both the miRNA/mRNA species of interest and controls, and the data obtained

were analyzed in concordance with the equation of Livak, Fold-change (FC) = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct\ (Ct_{miR\ target} - Ct_{miR26b/RUN44})_{tumoral\ tissue} - \Delta Ct\ (Ct_{miR\ target} - Ct_{miR26b/RNU44})_{normal\ tissue}$ (18). A fold change value below 1 indicates that the miRNAs are downregulated, whereas a value exceeding 1 means that the miRNAs are upregulated in the CRC relative to NATS. Thus, the results were expressed as FC in comparison with the calibrator sample, which was considered the normal value and was assumed to equal 1.

Statistical Analysis

Data obtained were analyzed and graphs were constructed using SPSS version 20.0 software (SPSS, Chicago, IL, USA) and MedCalc version 19.0.3 software (MedCalc, Ostend, Belgium). Differences between CRC and NATS were analyzed by the Wilcoxon test, while correlations between miRNAs expression levels as well as clinicopathological features were examined using the Mann-Whitney U test for two independent groups and Kruskal-Wallis H test for three independent groups. Survival rates for each miRNA were estimated using the Kaplan-Meier method and differences between low and high expression were calculated by using log-rank tests.

The diagnostic efficacy of selected miRNAs to function as prognostic biomarkers was evaluated by using Receiver operating characteristics (ROC). Similarly, the area under the curve was plotted (AUC) to assess to evaluate the power of selected miRNAs to functions as a diagnostic tool in order to discriminate CRC from NATS. Sensitivity and specificity were then defined by the optimal cut-off point, which refers to the maximized value of the area under the ROC (Youden index). The univariate prognostic analysis revealed the parameters which affected the prognosis of CRC patients, as miRNAs expression levels and clinicopathological characteristics.

Results

Demographic and clinicopathological characteristics of the cases

Among the 41 patients included in the study, 20 patients (48.7%) were male and 21 were female (51.2%); the average age ± SD of the group was 69.5 ± 8.53, with a range between 48 to 89 years. According to the histopathological examination, all patients included in the study were diagnosed with colon or rectum adenocarcinoma. The tumor site location was the proximal colon for 39% of patients (n = 16), the distal colon for 34.14% of cases (n = 14), and the rectum for 26.82% of cases (n = 11). Statistically, in the proximal colon, ulcer-vegetative lesions were predominated in 21.9% of cases, followed by ulcerated ones with 12.1% and polyploid with 4.87%. The distal colon was represented by ulcerated-infiltrative lesions in 17.0% of cases, ulcerated-vegetative in 7.31%, and polyploid in 2.43%.

Based on tumor differentiation grade, 21.95% (9/41) of cases were well-differentiated tumors, 73.18% (30/41) moderately differentiated, and 4.87% (2/41) poorly differentiated. In the proximal colon, moderately differentiated lesions were predominated (29.2%), followed by poorly differentiated (4.87%) and well-differentiated (2.43%), respectively; in the distal colon was involved in 17.0% of cases of moderately differentiated and well-differentiated adenocarcinomas (14.6%), respectively, while the rectum was represented by 21.9% moderate and 9.75% well-differentiated tumors.

Immunohistochemical analysis of nuclear proteins

IHC analysis was performed on 26 cases (63.41%) of CRC. The proteins hMLH1 and hPMS2 were not present in a 48-year-old woman (case 10) and a 71-year-old man (case 29), both cases with moderately differentiated adenocarcinoma of the proximal colon (cecum) and no history family of documented cancer. The expression of hPMS2 protein was negative in tumor tissues collected from a 77-year-old woman (case 14) with mucinous adenocarcinoma of the cecum, with a high grade of malignancy, and no documented family history of cancer.

Loss the expressions of proteins hMSH6 and hMSH2 was observed in a 74-year-old woman (case 33) and an 84-year-old man (case 34), both with moderately differentiated adenocarcinoma of the proximal colon (cecum). It can be summarized that all tumors were

localized in the proximal colon, and suppression of MSH6/MSH2 heterodimers was observed in 2 cases, while suppression of MLH1/PMS2 was observed in 3 cases.

Analysis of the miRNA genes expression

The average of ΔCt values was used to test the statistical difference between the two samples (NOR vs CRC) and the median values of relative expression (RQ) to find out how many times a selected gene is higher (overexpressed) or lower (underexpressed) in patients with CRC.

When the miRNA expressions were compared in the CRC relative to the NATS, five miRNAs (miR-21, -141, -182, -183, and -370) were found to be overexpressed, and eight (miR-30c, -144, -143, -145, -375, -214, -195, and -299) were found to be underexpressed (Tabel 1).

Among the miRNAs that were overexpressed in the CRC samples, miR-21 was overexpressed in 90% of cases (37/41; $P < 0.001$; figure 1A), miR-141 in 75% of cases (38/41; $P < 0.001$; figure 1B), miR-182 in 92% of cases (38/41; $P < 0.001$; figure 1C), miR-183 in 87% of cases (36/41; $P < 0.001$; figure 1D), and miR-370 in 87% of cases (36/41; $P < 0.001$, figure 1E). The mean FC level expressions of miR-182, miR-183, and miR-370 in the tumor samples as compared to the NATS were the most significantly overexpressed. Indeed, miR-182 was expressed by about 5.4 times, miR-183 by about 4.7 times, and miR-370 by about 4.0 times, whereas the miR-141 overexpression was only 1.69 times.

Quantification analyses were shown that levels of miR-30c, miR-144, miR-375, miR-214, miR-195, miR-143, miR-145, and miR-299 were significantly downregulated in CRC relative to NATS. Thus miR-30c was underexpressed in 78% of cases (32/41, $P < 0.001$; figure 1F), miR-144 in 63% of cases (26/41, $P = 0.02$; figure 1G), miR-375 in 78% of cases (32/41, $P < 0.001$; figure 1H), miR-214 in 70% of cases (29/41, $P = 0.04$; figure 1L), miR-299 in 70% of cases (29/41; $P = 0.20$), miR-143 in 80% of cases (33/41, $P < 0.007$; figure 1J), miR-145 in 85% cases (35/41, $P < 0.001$; figure 1K), and miR-195 in 90% of cases (37/41, $P < 0.001$; figure 2I).

The mean FC level expressions of miR-375, miR-195, miR-145, and miR-144 in tumor samples were the most underexpressed. Indeed, miR-375 was downregulated by about 19 times, miR-195 by about 4.6 times, miR-145 by about 21 times, and miR-144 was expressed 2.2 times less frequently in the tumor samples as compared to the NATS. The relative expression ratio for miR-299 suggested that it was also underexpressed in CRC by about 1.7 times; however, the statistical analysis did not reveal any significant differences.

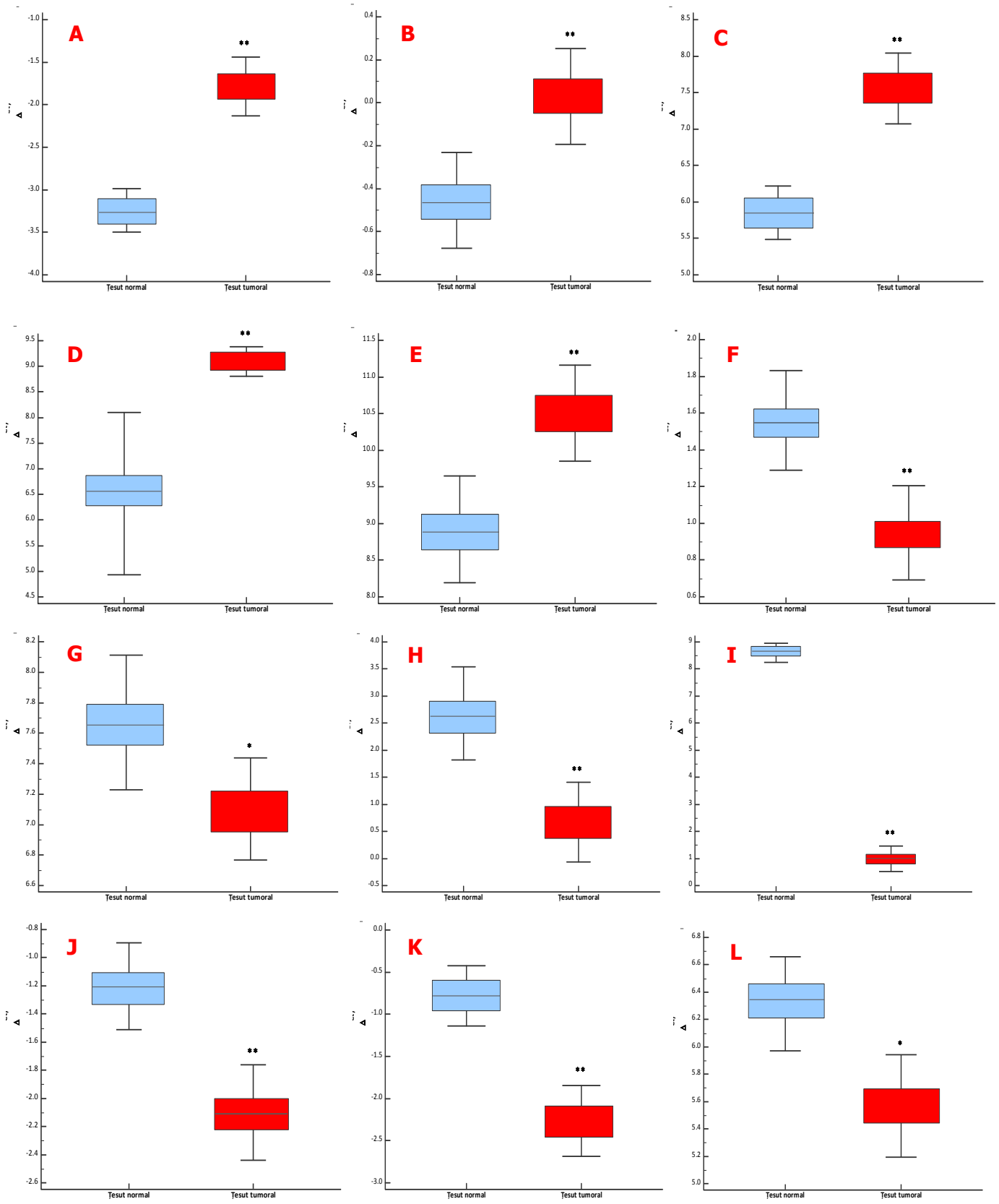

Figure 1. Expression of selected miRNA species (ΔCt) in colorectal tumor samples (CRC) relative to adjacent non-tumor samples (NOR).

Table 1. MiRNAs overexpressed or underexpressed in CRC relative to NATS.

miRNA	Normal tissue (ΔC_t)		Tumoral tissue (ΔC_t)			FC ($1/2^{-\Delta\Delta Ct}$)					
	Mean ± SD	Mean 95% CI	Mean ± SD	Mean 95% CI	P*	Lowest value	Maximal value	Median	Median 95% CI	FC	P**
miR-30c	1.55 ± 0.86	1.28 – 1.83	0.94 ± 0.81	0.69 – 1.20	< 0.001	0.42	8.43	1.50	1.25 – 1.96	1.80	< 0.001
miR-375	2.67 ± 2.72	1.81 – 3.53	0.67 ± 2.32	-0.06 – 1.40	< 0.001	0.10	155.41	2.93	1.74 – 4.81	19.01	< 0.001
miR-195	0.98 ± 1.50	0.51 – 1.46	8.60 ± 1.16	8.25 – 8.95	< 0.001	0.61	63.29	2.79	2.26 – 3.41	4.65	< 0.001
miR-143	-1.20 ± 1.41	-1.51 – -0.89	-2.09 ± 1.55	-2.44 – -1.75	< 0.001	0.15	46.62	1.58	1.31 – 3.00	5.07	< 0.001
miR-145	-0.78 ± 1.63	-1.14 – -0.42	-2.26 ± 1.91	-2.68 – -1.84	< 0.001	0.12	514.13	1.99	1.43 – 3.11	21.73	< 0.001
miR-214	6.31 ± 1.08	5.97 – 6.65	5.56 ± 1.18	5.19 – 5.94	0.047	0.20	6.87	1.41	1.19 – 1.87	1.96	< 0.001
miR-144	7.67 ± 1.39	7.22 – 8.11	7.10 ±1.05	6.76 – 7.43	0.026	0.32	15.42	1.14	0.97 – 2.58	2.25	< 0.001
miR-299	9.20 ± 1.14	8.79 – 9.61	8.94 ± 1.26	8.49 – 9.39	0.37	0.24	7.43	1.28	1.06 – 1.93	1.76	0.054
miR-21	-3.24 ± 0.81	-3.50 – -2.98	-1.78 ± 1.09	-2.12 – -1.43	< 0.001	0.03	13.96	3.34	2.62 – 4.63	4.19	< 0.001
miR-141	-0.45 ± 1.70	-0.67 – -0.23	0.02 ± 1.70	-0.19 – -0.25	< 0.001	0.45	5.51	1.33	1.15 – 1.44	1.69	< 0.001
miR-182	5.35 ± 1.16	5.48 – 6.21	7.55 ± 1.55	7.06 – 8.03	< 0.001	0.24	15.28	4.92	2.34 – 6.03	5.41	< 0.001
miR-183	6.51 ± 5.01	4.93 – 8.09	9.09 ± 0.89	8.81 – 9.37	< 0.001	0.13	24.81	4.70	2.41 – 5.46	5.52	< 0.001
miR-370	8.92 ± 2.05	8.19 – 9.64	10.50 ± 1.85	9.84 – 11.16	< 0.001	0.53	22.67	2.54	1.77 – 3.52	4.01	< 0.001

SD – Standard deviation; 95% CI – Confidenece interval; FC – Fold change; * Independent sample t-test; ** Signed rank-sum test.

Analysis of the mRNA genes expression

Analysis of gene expression patterns in the pathology of the colon and rectum neoplasm provides a global and detailed view of the architecture of the genes involved in tumorigenesis. The results of the study demonstrated that the mRNA genes were differentiated expressed in tumor tissue compared to adjacent normal mucosa. Indeed, the PTEN gene was downregulated in 65% of cases (27/41, P <0.001; figure 2A), MSH6 in 73% of cases, (30/41, P <0.002; figure 2B), and TP53 in 56% of cases (23/41, P <0.001; figure 2C), and both genes PIK3CA and mTOR was overexpressed in 70% of cases (29/41, P < 0.001; figures 2D and 2E).

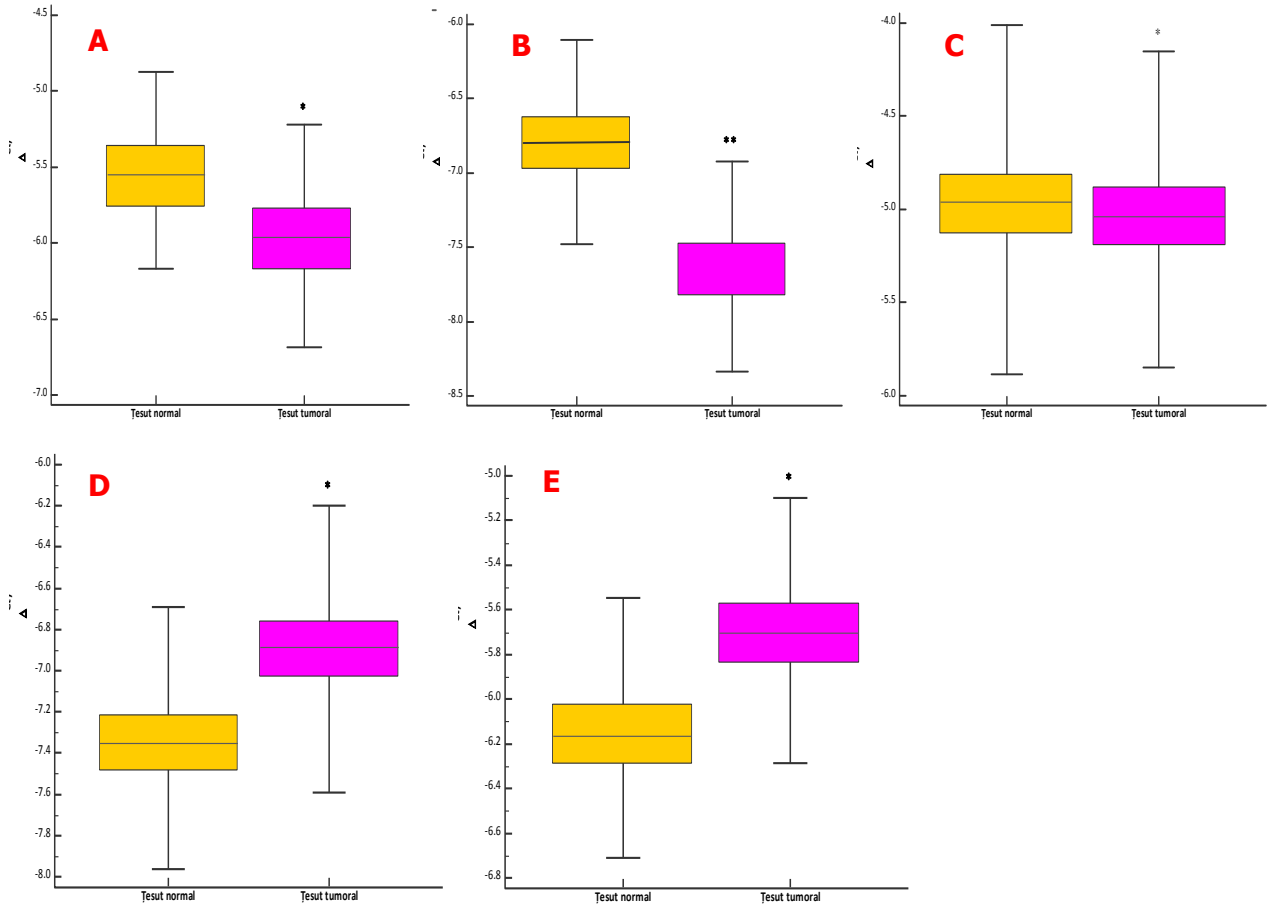

Figure 2. Expression levels of selected mRNA genes (ΔCt) in colorectal tumor samples (CRC) relative to adjacent non-tumor samples (NOR).

Diagnostic and prognostic feasibility of selected miRNAs genes

Receiver operating characteristic (ROC) and univariate and multivariate Cox-proportional hazard regression were used to determine the diagnostic and prognostic feasibility of selected miRNA to function as tisular biomarkers in CRC.

The diagnostic feasibility of the selected miRNA genes was evaluated by a representation two-dimensional of the ROC curves, using performance indicators such as sensitivity (real positive rate) and specificity (false positive rate) in different limit points (Youden index). The zone under the area of the ROC curve reflects the probability of correctly identifying tumor tissues from non-tumor tissues in patients with CRC.

Among the miRNAs with tumor suppressor functions, miR-30c (Figure 3A), miR-375 (Figure 3B), miR-195 (Figure 3C), miR-143 (Figure 3D), miR-145 (Figure 3E) , miR-144 (Figure 3F), miR-214 (Figure 3G) and miR-299 showed the following values of AUC, 0.36 (95% interval of confidence - CI: 0.51 - 0.73), 0.76 (0.69 - 0.82), 0.79 (0.69 - 0.87), 0.74 (0.67 - 0.81), 0.83 (0.73– 0.90), 0.63 (0.51–0.73) and 0.68 (0.57 - 0.78). In conclusion, it is observed that expression of miR-145 (Sensitivity - 82.93, Specificity - 85.37; P <0.001), miR-195 (Sensitivity - 78.05, Specificity - 78.05; P <0.001), miR-375 (Sensitivity - 75.61, Specificity - 80.49; P <0.001) and miR-143 (Sensitivity - 60.98, Specificity - 80.49; P <0.001) were able to distinguish tumor tissue from normal mucosal tissues with good specificity and sensitivity.

Regarding of miRNAs with oncogenic functions, miR-182 (Figure 3H), miR-183 (Figure 3I), miR-141 (Figure 3J), miR-21 (Figure 3K) and miR-370 (Figure 3L) presented the following values of AUC, respectively 0.91 (0.82 - 0.96), 0.86 (0.77 - 0.93), 0.68 (0.57 - 0.68), 0.73 (0.62 - 0.82) and 0.74 (0.67 - 0.80). Among all five oncomirs, miR-182 (Sensitivity - 87.80, Specificity - 90.24; P <0.001), miR-183, (Sensitivity - 75.61, Specificity - 92.68; P <0.001), miR-370 (Sensitivity - 65.85, Specificity - 76.83; P <0.001) and miR-21 (Sensitivity - 69.51, Specificity - 85.37; P <0.001) presented the best values of the performance indicators.

Moreover, in univariate and multivariate Cox-proportional hazard regression, nodal status, distant metastasis, miR-30c, miR-144, miR-375, miR-214, miR-21, miR-195, miR-141, miR-182, miR-183, and miR-370 were independent and significant predictor factors associated with CRC (Table 2).

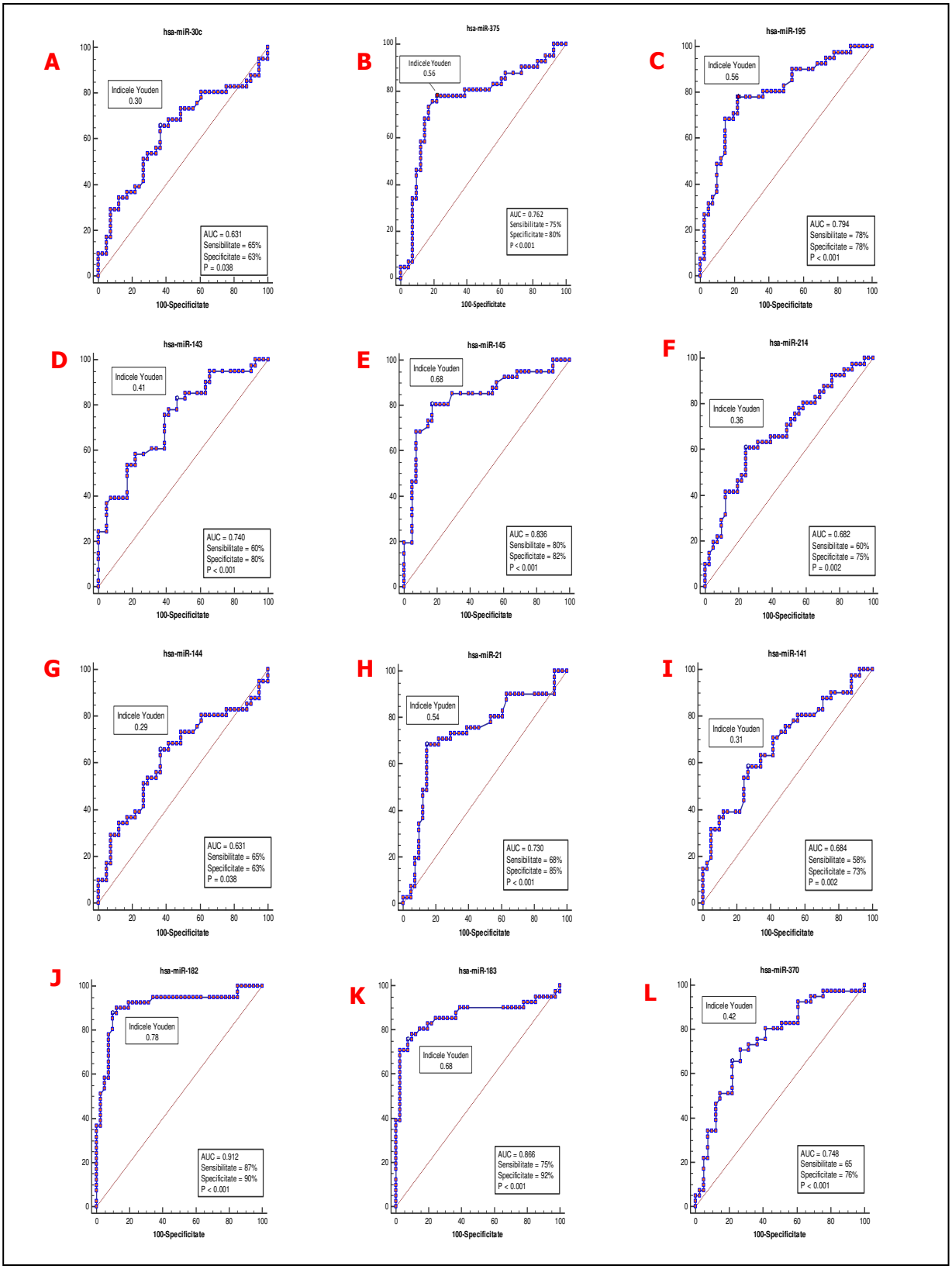

Figure 3. Diagnostic expression of selected miRNA genes among patients with CRC. In order to determine the diagnostic value of the selected miRNAs in the CCR vs NOR samples, the ROC analysis was performed and the area under the ROC curve (AUC) was graphically generated.

Table 2. Prognostic values of miRNAs genes and clinicopathological variables.

Clinicopathological variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI†	P	Hazard ratio	P	95% CI†
Age	1.65	0.73 - 3.98	0.32	–	–	–
Gender	1.23	1.07 - 3.50	0.32	–	–	–
Tumor location	1.52	1.28 - 3.79	0.63	–	–	–
Nodal status	1.23	1.26 - 3.86	0.049	–	–	–
Tumor invasion	2.83	0.88 - 7.29	0.035	3.86	0.012	0.77 - 3.90
Metastasis	6.32	0.79 - 1.17	0.023	3.29	0.035	1.00 - 3.54
Dimension	2.62	0.10 - 9.31	0.032	2.17	0.047	1.29 - 3.79
Hsa-miR-30c	3.62	1.02 - 5.31	0.032	4.97	0.012	1.02 - 5.24
Hsa-miR-144	3.23	0.81 - 5.06	0.028	3.31	0.016	0.80 - 5.51
Hsa-miR-375	3.22	0.65 - 5.90	0.012	2.06	0.035	0.88 - 5.22
Hsa-miR-214	3.95	0.54 - 5.88	0.023	4.90	0.014	1.00 - 3.54
Hsa-miR-21	3.76	1.00 - 5.20	0.001	1.88	0.042	1.29 - 3.79
Hsa-miR-195	2.86	0.82 - 5.56	0.001	2.20	0.034	1.24 - 3.86
Hsa-miR-141	2.41	0.79 - 6.17	0.035	1.00	0.023	0.80 - 5.51
Hsa-miR-182	2.32	0.10 - 7.31	0.035	3.98	0.023	0.88 - 5.22
Hsa-miR-183	2.42	0.73 - 4.97	0.023	4.50	0.042	0.76 - 6.14
Hsa-miR-370	2.53	1.02 - 4.31	0.011	3.79	0.031	1.75 - 5.62

† - Confidenece interval; P – statistical significance factor; HR – Hazard ratio.

Correlations between the expression of miRNAs/mRNA and clinicopathological features of CRC patients

Associations with clinicopathological characteristics were determined in order to explore the clinical relevance of selected miRNAs. In this context, age, sex, tumor size, tumor differentiation, tumor location, or microsatellite instability status, presented no statistically significant association (Table 3). Regarding tumor differentiation (stage G) a statistically significant association was observed with the low expression of the miR-30c gene, represented graphically in figure 4A (P = 0.02). Among all miRNAs studied the non-parametric Kruskal-Wallis and Mann-Whitney tests indicated a significant association between miR-183, miR-182, miR-141, miR-143, miR-145, miR-30c, miR-370, miR-144 and miR-21, and mRNA genes: PTEN and TP53 with some clinicopathological variables.

Regarding to the depth of tumor invasion, we observed a statistical association with the higher expression of the genes miR-182 (P <0.001), miR-183 (P <0.001), miR-21 (P <0.001) and miR-370 (P = 0.04), graphically represented in figure 4B, respectively with the low expression of miR-143 (P = 0.01), miR-145 (P = 0.04) and miR-144 (P = 0.04), graphically represented in figure 4C. Also, the low expression of the PTEN (P <0.001) and TP53 (P <0.001) genes were statistically significantly correlated with the stage of tumor invasion, figure 4D.

In addition, lymph node involvement, pN staging was statistically significantly correlated with higher expression of the miR-182 (P = 0.003), miR-183 (P = 0.03), miR-21 (P = 0.004), and miR-370 (P = 0.00) genes. 0.03), figure 4E, respectively with the low expression of the genes miR-143 (P = 0.03), miR-145 (P <0.001) and TP53 (P = 0.003), graphically represented in figure 4F.The advanced stage of distant metastasis showed higher values for the expression of genes miR-182 (P <0.001), miR-183 (P <0.001), miR-141 (P <0.001) and miR-370 (P <0.001), figure 4G, and lower values for reduced expression of miR-143 (P <0.001), miR-145 (P <0.001), miR-144 (P <0.001) and miR-375 (P <0.001), respectively, Figure 4H.

Moreover, we established a statistical association between the differentiated expression of miRNAs genes and the expression of mRNA genes. In this context, using Spearman analysis we observed that the expression of the PTEN gene was negatively correlated with miR-21 gene expression (rho = -0.34; P = 0.02), and the low expression of the TP53 gene was correlated inversely with the overexpression of miR-21 gene (rho = −0.49; P = 0.001).

Tabel 3. Clinicopathological variables of the cases ([§]Mann-Whitney; [ǂ]Kruskal-Wallis; *P < 0.05, **P< 0.01).

miARN species	Variables					TNM Staging			
	Age[§] ≤ 65 (26.2%) >65 (71.4%)	**Gender[§]** Masculin (48.8%) Feminin (51.2%)	**Location** Proximal (36.5%) Distal (26.8%) Rect (34.1%)	**Dimension[§]** ≥ 5 (53.6%) < 5 (46.3%)	**Grading[ǂ]** G1 (21.9%) G2 (73.1%) G3 (4.8%)	**pT[ǂ]** T2 (14.6%) T3 (41.4%) T4 (43.9%)	**pN[ǂ]** N0 (39.0%) N1 (29.2%) N2 (31.7%)	**pM[§]** M0 (63.4%) M1 (36.5%)	**MSI[§]** MSI S (80.7%) MSI I (12.1%)
miR-30c	0.43-0.93; P = 0.66	0.43-0.93; P = 0.60	19.69-22.43-21.09; P = 0.82	0.43-0.93; P = 0.47	30.33-20.93-10.20; P = 0.02*	21.67-22.47-19.39; P = 0.47	23.0-22.0-17.62; P = 0.45	0.43-0.93; P = 0.51	0.43-0.93; P = 0.0616
miR-375	0.10-0.91; P = 0.89	0.10-0.91; P = 0.79	21.31-19.21-22.82; P = 0.75	0.10-0.91; P = 0.42	20.83-20.0726.80; P = 0.50	15.33-21.06-22.83; P = 0.41	20-21.67-21.62; P = 0.91	0.10-0.91; P < 0.001**	0.10-0.91; P = 0.07
miR-195	0.22-0.71; P = 0.77	0.22-0.71; P = 0.71	21.69-22.07-18.64; P = 0.74	0.22-0.71; P = 0.51	20.17-22.27-14.40; P = 0.39	25.50-22.53-18.06; P = 0.33	24.25-19.42-18.46; P = 0.37	0.22-0.71; P = 0.87	0.22-0.71; P = 0.17
miR-144	0.35-1.12; P = 0.51	0.35-1.12; P = 0.55	23.12-17.36-22.55; P = 0.37	0.35-1.12; P = 0.45	18.83-20.50-26.60; P = 0.51	31.33-17.0-21.33; P = 0.04	25.94-16.08-19.46; P = 0.08	0.35-1.12; P < 0.001**	0.35-1.12; P = 0.10
miR-214	0.46 –0.14; P = 0.64	0.46 –0.14; P = 0.66	17.75-23.00-23.18; P = 0.38	0.46 –0.14; P = 0.26	24.83-20.83-17.40; P = 0.58	27.33-23.00-17.00; P = 0.12	25.19-16.08-20.38; P = 0.13	0.46 –0.14; P = 0.64	0.46 -0.14; P = 0.44
miR-299	0.21-0.83; P = 0.92	0.21-0.83; P = 0.42	17.94-20.17-19.33; P = 0.86	0.21-0.83; P = 0.32	16.0-19.07-23.00; P = 0.60	24.80-20.56-15.63; P = 0.18	23.50-11.45-20.67; P = 0.12	0.41-1.29; P = 0.79	0.41-1.29; P = 0.07
miR-143	0.19-0.66; P = 0.51	0.19-0.66; P = 0.50	15.81-24.00-24.73; P = 0.84	0.19-0.66; P = 0.10	26.50-20.23-19.00; P = 0.46	28.67-24.4715.17; P = 0.01*	25.31-22.83-14.0; P = 0.03*	0.21-0.83; P < 0.001**	0.21-0.83; P = 0.30
miR-145	0.19-0.66; P = 0.61	0.19-0.66; P = 0.67	18.00-21.93-24.18; P = 0.39	0.19-0.66; P = 0.27	25.67-21.17-14.40; P = 0.29	26.67-24.41-15.89; P = 0.04*	24.06-26-12.62; P < 0.001**	0.19-0.66; P < 0.001**	0.19-0.66; P = 0.40
miR-21	2.13-6.12; P = 0.67	2.13-6.12; P = 0.61	20.37-21.14-20.45; P = 0.90	2.13-6.12; P = 0.11	15.83-20.97-27.40; P = 0.28	9.33-17.35-28.33; P < 0.001**	15.80-18.17-30; P = 0.004**	2.13-6.12; P < 0.001**	2.13-6.12; P = 0.08
miR-141	1.03-1.83; P = 0.34	1.03-1.83; P = 0.74	24.25-17.00-21.36; P = 0.25	1.03-1.83; P = 0.54	26.33-19.17-25.60; P = 0.26	17.50-19.59-23.50; P =0.46	18.44-22.92-22.38; P = 0.54	1.03-1.83; P = 0.34	1.03-1.83; P = 0.34
miR-182	1.90-7.09; P = 0.58	1.90-7.09; P = 0.68	20.88-17.86-25.18; P = 0.31	1.90-7.09; P = 0.48	20.50-21.07-21.20; P = 0.99	7.33-16.82-29.50; P < 0.001**	13.69-22.83-28.31; P = 0.003**	1.90-7.09; P < 0.001**	1.90-7.09; P = 0.37
miR-183	1.19-7.48; P = 0.88	1.19-7.48; P = 0.98	19.81-19.32-24.86; P = 0.45	1.19-7.48; P = 0.58	22.92-19.73-26.30; P = 0.47	6.58-20.29-26.47; P < 0.001**	16.22-20.25-27.58; P = 0.03*	1.19-7.48; P < 0.001**	1.19-7.48; P = 0.06
miR-370	1.37-6.04; P = 0.82	1.37-6.04; P = 0.81	16.62-23.07-24.73; P = 0.16	1.37-6.04; P = 0.18	18.17-21.40-22.00; P = 0.81	13.83-18.24-26.0; P 0.04*	18-17.33-28.08; P = 0.03*	1.37-6.04; P < 0.001**	1.37-6.04; P = 0.70

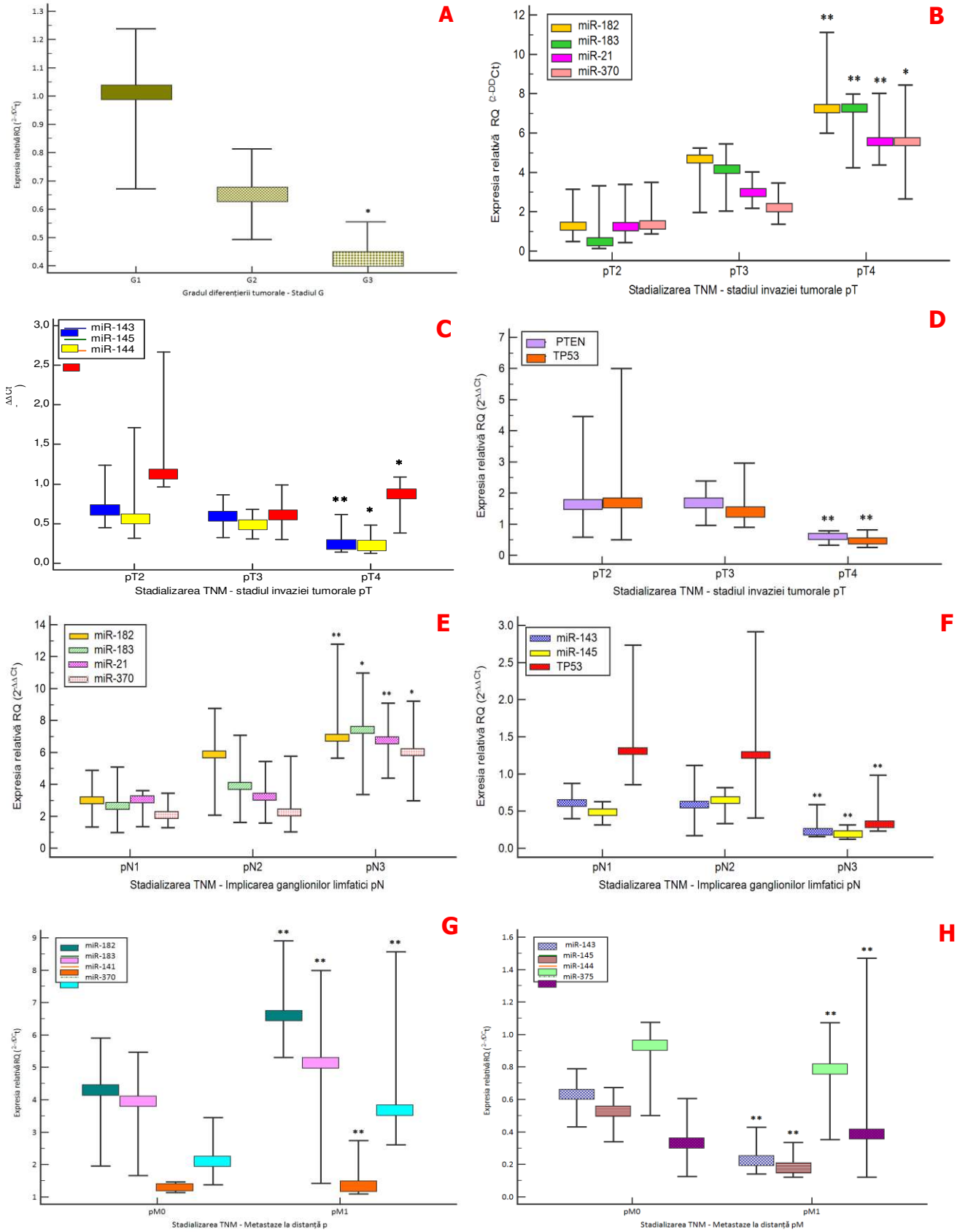

Figure 4. Fold change expression of selected miRNA/mRNA genes relative to a different stage of CRC.

Discussions

The interdependence relationship between molecular imbalances and tumor progression postulated by Vogelstein and Fearon in 1990 has been for two decades the paradigm of genetic and epigenetic events encountered in colorectal cancer, being an example of pathophysiological evolution for many other cancers (9). Nowadays this model is considered far too simplistic in light of recent studies that suggest an over 90% of the human genome is dynamically and persuasively transcribed into other RNA species than mRNA, namely as noncoding RNAs (19).

Among all types of ncRNAs, microRNAs have received particular attention and have been proposed as useful diagnostic and prognostic biomarkers. In CRC, a large variety of miRNAs are up- or down-regulated as compared to normal tissues. MiRNAs that are consistently found to be down-regulated in CRC act as tumor suppressor genes and are accordingly termed "mirsupps". In contrast, miRNAs that are consistently found to be upregulated in CRC act as oncogenes and are referred to as "oncomirs" (20). Against this backdrop, studying the specific function of miRNAs in human carcinogenesis will help characterize new targets for cancer research, diagnosis, and treatment of cancer at the molecular level.

In concordance with previously mentioned, in this paper, we demonstrated that the expression profile of miRNA molecules, respectively mRNA, was systematically significantly modified in the selected group of Romanian patients with CRC.

This was realized using the qRT-PCR two-step method and TaqMan®MGB miRNA/mRNA-specific hydrolysis probes. This study included 41 patients diagnosed and confirmed histopathologically with colon or rectum adenocarcinoma. Specimens included two types of tissue fragments collected immediately after surgery, one sample from tumor component, and one from normal adjacent mucosa (harvested > 5 cm from tumor tissue). Exploration and clinical significance of selected genes were performed on a panel of 14 miRNA genes (miR-30c, -375, -195, -144, -214, -143, -145, -299, -21, -182, -183, - 370 and -21) and 5 mRNA genes (PTEN, MSH6, PIK3CA, mTOR and TP53), genes were selected based on a comprehensive literature review, previously published data and by consulting the Sanger Center Registry and miRBase databases.

In this regard, 13 miRNA genes were statistically significantly abnormally expressed in CRC compared to NOR, of which 5 miRNAs were overexpressed and 8 underexpressed. The overexpressed miRNAs consist of miR-141, -182, -183, -370, and -21. In contrast,

underexpressed miRNAs are represented by miR-30c, -144, -375, -214, -299, -195, -143 and -145 species. Overexpression of miR-182, -183, -370, and subexpression of miR-30c, -375, and -195 presented the most significant changes in expression and one miRNA did not reveal any statistical difference between the two samples, respectively miR-299. Moreover, the expression of mRNA genes is expressed statistically differentiated in CRC compared neither to NOR, with 3 underexpressed (MSH6, TP53, and PTEN) and 2 overexpressed (mTOR and PIK3CA). In this context, our findings demonstrate that altered expression of selected miRNA genes plays a pivotal role in colorectal adenocarcinoma and their expression is directly influenced by the cellular microenvironment. The inadequate expression of miRNAs participates actively and constantly in the development of colorectal cancer by altering the levels of oncogenic or tumor suppressor genes transcription and in accordance with this could be participated in the evolution of CRC (21-26).

Furthermore, in this thesis, we demonstrated that the differentiated expression of the miRNAs is correlated with different clinical and histological variables, such as tumor differentiation, stage of tumor invasion, the involvement of lymph nodes, or appearance of distant metastases. In this regard, non-parametric tests indicated a statistically significant tendency of association between the depth of tumor invasion and higher expression of genes miR-182, miR-183, miR-21, miR-370, and lower expression of miR-143, respectively. MiR-145, miR-144, PTEN, and TP53. In addition, we observed a gradual increase in fold change expression of the genes miR-182, miR-183, miR-21, miR-370, and respectively a decrease in the expression of the miR-143, miR-145, and TP53 genes, expressions which were significantly associated with the involvement of loco-regional lymph nodes.

The advanced stage of distant metastasis, it showed higher values for the expression of genes: miR-182, miR-183, miR-141, and miR-370, and respectively lower values for the expression: miR-143, miR-145, miR -144, and miR-375. All these findings are consistent with previously published studies, suggesting that the studied miRNAs show the same pattern of expression among Romanian patients, and their expression is not influenced by unique genetic or epigenetic imbalances of the population or socioeconomic factors associated with each region (25-28).

miRNAs have some features that make them attractive as biomarkers of malignancy, offering new opportunities for improving diagnosis, prognosis, and the management of CRC.

Their suitability includes the altered expression of miRNAs in malignant versus normal tissue, their ability to resist degradation by endogenous ribonuclease, their ease of quantitation using several methods (e.g., qRT-PCR, microarray or sequencing technology), and especially their differentiated expression in different types of tumor.[54] Furthermore, the ROC curve analysis demonstrated that miR-182, -183, -21, -143, -145, -195 and -375 are useful tools for differentiating between tumor samples and normal adjacent tissues in CRC patients, with a sensitivity of between 87.80 – 60.98% and a specificity between 92.68 – 78.05%, suggesting the clinical relevance of these biomarkers. In univariate and multivariate analysis expression levels of the miR-30c, miR-144, miR-375, miR-214, miR-21, miR-195, miR-141, miR-182, miR-183, miR-370, distant metastases, and tumor size were independent and significant predictor factors associated with CRC.

Although the expression level was not determined in the blood, we have identified a comprehensive list of genes with consistent expression patterns in colorectal adenocarcinoma, genes that could serve as potential molecular markers complementary to existing histopathological factors.

Conclusion

The summary of the thesis demonstrates that the expression of the analyzed miRNAs is systematically modified in our cohort of patients. Furthermore, altered expression of these miRNAs varies in different stages of CRC development and may serve as potential diagnosis molecular biomarkers in Romanian patients with CRC. Moreover, the observations of the doctoral thesis reinforce the previous studies and offer a justification in the application of molecular histopathology in CRC.

In conclusion, the diagnostic value of the studied miRNA and mRNA genes has a significant impact on the accuracy of the diagnosis in Romanian patients with colorectal adenocarcinoma, but are not yet satisfactory markers able to concur with routine tests, but offer promising prospects and open new opportunities in diagnosis. Therefore, further investigations are needed to confirm our findings.

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Research articles

- **Costel Brînzan**, Mariana Aşchie, Elena Matei, Anca Mitroi, Georgeta Cozaru. Molecular expression profiles of selected microRNAs in colorectal adenocarcinoma in patients from the south-eastern part of Romania. Medicine 2019 Nov;98(47):e18122. doi: 10.1097/MD.0000000000018122.(https://journals.lww.com/mdjournal/FullText/2019/11220/Molecular_expression_profiles_of_selected.84.aspx).

- **Costel Brînzan**, Mariana Aşchie, Cătălin Nicolae Grasa, Anca Mitroi, Elena Matei, Georgeta Cozaru. The mutation profiles of KRAS and BRAF genes in Romanian colorectal cancer cohort. Rev.Chim 2019;70(4):1346-1350.

  (https://revistadechimie.ro/Articles.asp?ID=7124)

- **Costel, Brînzan**; Mariana, Aschie; Georgeta, Cozaru; Eugen, Dumitru; Anca, Mitroi. *The diagnostic value of miR-92a, -145 and -145 expression levels in patients with colorectal adenocarcinoma from Romania,* Medicine (în cur de publicare).

Presentations to Learned Societies and Published Abstracts

Costel Brînzan, Mariana Aşchie, Georgeta Cozaru, Elena Matei, Anca Florentina. Expression patterns of miR-143, -145 and -92a in colorectal cancer from south-east Romanian patients. The 11th National Congress with international participation of the Romanian Society of Cell Biology 2019 Jun.

- M Aşchie, I Poinareanu, GC Cozaru, AF Mitroi, **C Brînzan**, D Aşchie, GI Baltatescu, M Enciu, AA Nicolau, A Chisoi, O Cojocaru, Genotyping-RAS mutations analysis in colorectal cancer, VIRCHOWS ARCHIV, SPRINGER, S198-S198, volum 471, 2017/9/1, IF = 2,848.

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