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Exosome-Carried microRNA-375 Inhibits Cell Progression and Dissemination via Bcl-2 Blocking in Colon Cancer

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ABSTRACT

Background & Aims: Worldwide, colorectal cancer (CRC) is the third most common cancer in men and second in women. The aim of the current study was to identify whether the miR-375 is indeed down-regulated in metastatic CRC and if it could be considered as a potential minimally invasive prognostic biomarker for CRC.
Methods. Exosomes were isolated and characterized from patients with liver metastasis from CCR. The characterization of exosome was performed using TEM/SEM. HCT116 cells were treated with miR-375 mimic, NSM and miR-375 inhibitor. Functional assays included cell counting assay for 14 days, Matrigel invasion assay, apoptosis assay by flow cytometry using Annexin V-FITC, RT-PCR and Western blotting.
Results. Increased proliferation potential was proven for the cells transfected with miR-375 inhibitor, while the miR-375 mimic decreased the cell number. The cells transfected with the miR-375 inhibitor are aggressive and cross the membrane; 3.84% of the cells transfected with the miR-375 inhibitor entered apoptosis, while 6.45% of those transfected with the non-specific mimic were in programmed cell death, less than those transfected with the microRNA. RT-PCR for Bcl-2 expression showed that Bcl-2 is down-regulated for miR-375 inhibitor and up-regulated for the miR-375 mimic, a result confirmed by Western blotting.
Conclusion. The present study brings to the forefront new data that suggest miR-375 as a new player in controlling the pathways responsible for inhibiting the natural history of CRC tumor cells, via the Bcl-2 pathway.

Key words: microRNA-375, colon cancer, BCL-2 pathway, biomarkers.

Abbreviations: APC: adenomatous polyposis coli; CRC: colorectal cancer; EGFR: epidermal growth factor receptor; ERB: Erythroblastic Leukemia Viral Oncogene Homolog; FITC: Fluorescein isothiocyanate; MAPK: mitogen-activated protein-kinase; miR: microRNA; mTOR: mammalian target of rapamycin; NSM: non specific mimic; qRT-PCR: quantitative real time polymerase chain reaction; SEM: scanning electron microscopy; TEM: transmission electron microscopy; TGF-beta: transforming growth factor beta.

INTRODUCTION

Worldwide, colorectal cancer (CRC) is the third most common cancer in men and second in women. In 2008, approximately 600,000 deaths (8%, third most diagnosed cancer) were related to CRC of all cancer deaths [1]. Understanding the molecular mechanisms that govern tumor growth and metastasis is imperative for individualized medicine as well as for future treatment developments. Molecular analysis has enabled the development of diagnostic and therapeutic tools that offer the possibility of individual tailored medicine that previously has been unavailable [2, 3]. There is increasing evidence for the fact that a class of small non-coding RNAs, called microRNAs (microRNAs, 18–25 nucleotides in length, endogenously expressed, with post-transcriptional gene-expression regulation activity by binding to 3' untranslated region - 3'UTR, circulating via exosomes) could be key regulators for different basic biological processes (including tumor initiation and progression via alterations in the development, cellular differentiation, proliferation, and apoptosis processes) by tightly regulating gene expression [4-6]. To the present day, multiple studies have concluded that microRNAs can have also a tumor suppressor role as they can function as oncogenes [4-6, 14]. Different cancer pathologies show unique miRNA signatures: however, the prognostic value

of microRNAs in metastatic cancers has just been started to be investigated [7, 8]. The number of verified microRNAs is constantly growing; recent studies have focused upon the value of microRNAs especially in metastatic colon and rectal cancers. By understanding the molecular and genetic mechanisms by which a carcinoma in situ is able to invade through the basal membrane, grow and even disseminate in distant organs evading the host immune system and exogenous systemic chemotherapy, healthcare providers can develop valuable and applicable future treatments for CRC. The epigenetic events that correlate with a positive feed-back between a cancer cell and the surrounding fibrous tissue may hold the key answer for many questions regarding early detection and improved management for CRC [8]. Exosomes are considered to be miR-carriers as they offer increased stability for cell-free microRNAs in various bodily fluids and thus enable these nucleic acids to be efficient biomarkers for the non-invasive diagnosis and prognosis of CRC. From the management, follow-up and prognosis point of view cancers of the colon and rectum should be distinguished one from the other and considered two different entities.

In a recent study, Gaedcke et al. [7] have identified at least 49 distinct microRNA significantly expressed profiles in rectal cancer, out of which 28 were already described for colon cancer [9]. MiR-21, miR-31, miR-135b, miR-223 (expressed both in colon and rectal cancer) were found to be all up-regulated, whereas miR-195, miR-378, miR-192–194–215, miR-143–145, miR-1, and members of the miR-30 and let-7 family (both for colon and rectum) were all down-regulated. Therefore, the afore mentioned study concludes that the remaining 21 miRs could consist of rectal-cancer-specific molecular profiles (miR-492, miR-542-5p, miR-584, miR-483-5p, miR-144, miR-2110, miR-652, and the C/D box snoRNA, SNORD12B – up-regulated, MiR-375, miR-147b, miR-148a, miR-190, miR-26a/b, miR-29c, and miR-338-3p – down-regulated). MiR-143, miR-145 (known tumor-suppressor genes), miR-21 and miR-31 (known to be oncogenes) are the most consistently reported to have dysregulated expression in CRC [15-18]. Colorectal cancer tumorigenesis miR-targeted/regulated genes/pathways/networks include MYC, APC, ERBB2-ERBB3, ZEB1, FOXD3, Wnt/ beta-catenin, TGF-beta, mTOR, insulin, mitogen-activated protein-kinase (MAPK), EGFR/KRAS and ErbB signaling [7, 10, 11, 18]. The same authors found one miR that has not been associated with colon cancer before their study - miR-375 - the single most down-regulated miRNA in rectal cancer (3-fold change). It is known that concomitant to miR-148a and miR-190, mir-375 targets BCL2; the role of miR-148a and miR-190 role in CRC and pancreatic cancer tumorigenesis was previously described [19-21].

For the development and progression of CRC, miR-375 targets genes that play an important role in regulating pathways, such as MAPK, Wnt, TGF-beta signal pathways [15]. In most CRC tumors (90%) a regulatory factor of the Wnt/β-catenin signaling pathway is mutated [the Wnt ligand which initiates signaling through the Frizzled (FZD) receptor is one of the known targets of miR-375] [15, 19]. Several studies identified miR-375 to be down-regulated in CRC, without mentioning its role for liver metastasis progression [22, 23], but underlying its capacity to inhibit primary tumor growth by targeting PI3K/Akt signaling pathway [24] and by stimulating apoptosis by targeting YAP1 [25]. To summarize, more and more data show that a down-regulated miR-375, either found in primary tumor specimens or via exosomes in circulating fluids, may subclinically identify the presence or the persistence of cancer, underlining a poor prognosis in many solid tumors [26, 27]. No data was found in relation to miR-375 and Bcl-2 blocking, responsible for tumoral progression and dissemination.

The aim of the current study was to identify whether the miR-375 is indeed down-regulated in metastatic CRC, whether it has potential Bcl-2 blocking action and if it could be considered as a potential minimally invasive prognostic biomarker for CRC by inhibition of malignant proliferation and dissemination.

MATERIAL AND METHODS

Exosome isolation and characterization

Exosomes have been isolated according to the protocol described by Li et al. [28] from patients diagnosed with liver metastases from CCR at the Ion Chiricuta Oncology Institute in Cluj Napoca, Romania. The patients' serum was obtained previous to chemotherapy and a written consent was signed according to all legal and ethical laws of the European Union and according to the Declaration of Helsinki.

ExoQuick exosome precipitation solution kit (System Biosciences, Mountain View, California, USA) was used to isolate the membrane vesicles from 250 μl of serum. The serum was centrifuged at 3000 x G for 15 minutes in order to remove all cellular debris before being transferred to a sterile vessel and mixed with the exosome precipitation solution. Afterwards, the ExoQuick/plasma mixture was again centrifuged at 3000 x G for 30 minutes at room temperature, the supernatant was aspirated and the residual solution again centrifuged at 3000 x G for 5 minutes. The exosome pellet obtained was later re-suspended in sterile phosphate buffer solution (PBS). Characterization was done according to our previously described protocol [29], using both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Slides were prepared for TEM according to the usual protocols [30, 31]. Prefixation was done for 2 hours in 2.7% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4), post-fixation for 1.5 hours in 2% osmium tetroxide (in 0.15 M phosphate buffer, pH 7.4), dehydration with five solutions of acetone of increasing concentrations (between 50–100%, 15 minutes each), infiltration with solutions of Epon 812 in acetone (1:2, 1:1, 2:1, 1 hour each, and in pure Epon overnight) and embedding in Epon 812 (72 hours at 600 C). The ultrathin sections of 70–80 nm thickness were obtained on a LKB 8800 ULTROTOME® III ultramicrotome (LKB, Bromma, Sweden), using a DiATOME diamond knife (Diatome Ltd., Bienne, Switzerland), then stained with uranyl acetate and lead citrate. Sections were examined in a JEOL JEM 100CX II transmission electron microscope (Jeol Ltd. Tokyo, Japan) at 100 kV acceleration voltage and magnifications between 3600× and 19 000×. The most representative images were photographed on 4489 Kodak electron microscope films (Carestream Health Inc., New York, USA), and the films were scanned in an Imacon Flexitight X5 film scanner (Hasselblad Imacon, Copenhagen

Sweden), using the computer software Imacon FlexColor X5 (Hasselblad Imacon, Copenhagen Sweden). For the *in vitro* internalization of the exosomes, we used the HCT-116 cell line (ATCC- American Type Culture Collection, Manassas, Virginia, USA). Briefly, the exosome pallet was mixed with the cells in culture and then slides were done in order to prove the internalization of the multivesicular bodies. The characterization of the exosomes is carried out, apart from using electron microscopy, by Western blotting for the exosome-specific markers Tsg-101 and CD 81, as previously described by our team [28, 29]. The protocol for Western blotting is detailed later in the paper.

Cell culture protocols

HCT 116 cells were grown in a humidified atmosphere at 37°C air, 95%; carbon dioxide (CO2), 5%. The culture medium was RPMI 1640 medium, with 10% fetal bovine serum (FBS) and 1% antibiotics. Cell passage and culture was carried out as previously described [32, 33].

DNA and RNA extraction

Total RNA was isolated using TRIzol reagent (Invitrogen), as previously described [34].

Transfection of miR-375 inhibitor, miR-375 mimic and nonspecific mimic (NSM)

Synthesized RNA duplexes of miR-375 inhibitors and mimics, as well as the non-specific mimic (NSM) were purchased from Dharmacon (Lafayette, Colorado, USA). 30–50% confluent cells were transfected with 60 nM of miR-375 inhibitor or inhibitor negative control using Lipofectamine RNAi MAX (Invitrogen, Waltham, Massachusetts, USA). RNA and proteins were harvested 72 hours after transfection.

Cell counting

15,000 cells were plated in 24-well plates (Day 0), transfected after 24 hours and counted at days 1, 2, 4, 7, 10 and 14 by using both a hemocytometer and a Leica S80 inverted phase microscope, as well as the Countess Automated Cell Counter (Invitrogen).

Flow cytometry

5×10^5 cancer cells were transfected with 60 nM mimics or inhibitors of microRNA-375 or non-specific controls, respectively. After 48 hours, cells were double stained with PI and annexin-V (Vybrant Apoptosis Assay Kit, Invitrogen). Fluorescence intensity was measured using a flow cytometer (BD FACS Canto II) to assess early apoptotic cells, defined as those staining only with annexin-V.

Cell invasion assay

Scratch cell migration and Matrigel invasion assay to estimate the *in vitro* invasion ability of both parental and transfected cancer cells. Serum-free RPMI1640 medium was mixed with Matrigel (1:10; BD Biosciences, Bedford, Massachusetts, USA). The bottom of the culture inserts (8-μm pores) in 24-well tissue culture plates (Transwell, Corning, Corning, NY, USA) was coated with 50 μl of the mixture. Afterwards, the Matrigel was allowed to solidify at 37 °C for 4 h. After solidification, 5×10^4 cells were harvested by trypsinization, washed with serum-free medium then placed in the upper chamber. The lower chamber contained 10% fetal bovine serum used as a chemoattractant. After 48h of incubation at 37 °C with 5% CO2, cells in the inner side of the chamber were removed using cotton swabs. The number of cells that invaded to the basal side of the membrane was quantified by counting 16 independent symmetrical visual fields under the microscope. Cell morphology was observed after staining with 0.1% Crystal violet.

Quantitative qRT-PCR (qRT-PCR) for miR and mRNA expression

miR qRP-PCR was performed to confirm the expression of miR-375. TaqMan MiR Assays (Human Applied Biosystems, Foster City, CA, USA) were used. Cycle passing threshold (Ct) was recorded and normalized to RNU6B expression. Relative expression was calculated as $2^{Ct\_miR\text{-}375\text{-}Ct\_RNU6B}$. PCR reactions were carried out in duplicate. All mRNA qRT-PCR values were normalized to beta-actin and the relative expression was calculated as $2^{Ct\_target\ gene\text{-}Ct\_beta\text{-}actin}$. PCR reactions were carried out in duplicate.

Western blotting

Cells were lysed in Laemmli sample buffer (Bio-Rad, Hercules, California, USA) supplemented with a protease inhibitor complete EDTA-free (Roche). Protein concentration was measured using BCA Protein Assay kit (Pierce, Rockford, Massachusetts, USA). Cell lysates (50 μg) were electrophoresed on 10–20% polyacrylamide gells (Bio-Rad) and transferred to ImmobilonPSQ membranes (Millipore, Bedford, Massachusetts, USA). The membranes were blocked with TBS containing 5% skim milk and 0.1% Tween-20, then incubated with the primary antibody. Antibody to Bcl-2 was purchased from Abcam (Cambridge, United Kingdom). The membranes were incubated after washing with HRP-conjugated goat anti-rabbit IgG (Calbiochem, Gibbstown, New Jersey, USA) and analyzed using enhanced chemiluminescence-plus reagent (GE Healthcare, Buckinghamshire, United Kingdom). Densitometry was performed on the western blot images by using the J-Image software (http://rsbweb.nih.gov/ij/).

Statistical analysis

The statistical analysis was performed using R (R Development core team, USA) and GraphPad Prism 5.0 (GraphPad Software INC., CA, USA). The obtained data was fist examined for normality of distribution using the Shapiro-Wilk test. The distribution of all the obtained data was Gaussian, thus it was analyzed using a parametric test (Two-Way ANOVA with Turkey post-test). The differences were considered significant when $P < 0.05$.

RESULTS

Exosome characterization

The multivesicular bodies isolated were characterized using electron microscopy. Thus, SEM was used to assess the shape of the exosomes, as seen in Fig. 1. The role of exosomes in intercellular communication was assessed by using the

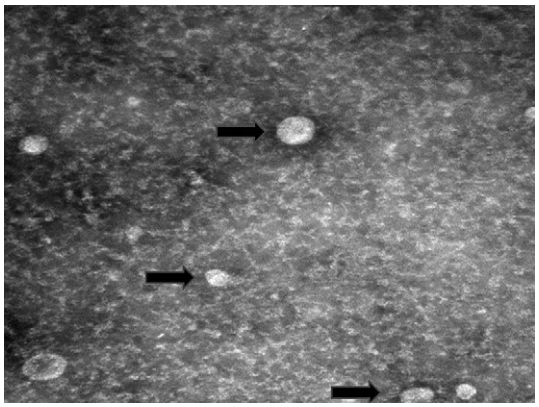

Fig. 1. Scanning electron microscopy image of the exosomes.

exosomes isolated from CCR patients in culture on the HCT116 cells. The exosomes were internalized by the malignant cells as seen in Figs. 2A and B, images obtained by TEM. In Fig. 2A we can clearly see vesicular bodies on the external surface of the cell membrane. In Fig. 2B, the vesicle was internalized and can be seen inside the cell, surrounded by the rough endosplasmatic reticulum. Figure 2C presents the complete characterization of exosomes using western blotting. The investigated proteins were Tsg101 and CD81, previously described by our team as exosome-specific markers. We investigated the levels for these two proteins in both exosomes isolated from our patients, as well as from cells in culture, as negative controls. Thus, in the left images we can clearly see that the multivesicular bodies isolated by us indeed express these markers. In the right images, we attached the western blotting for β-actin, as a positive control. The protein levels were very similar between the samples, proving that our protocol was accurate.

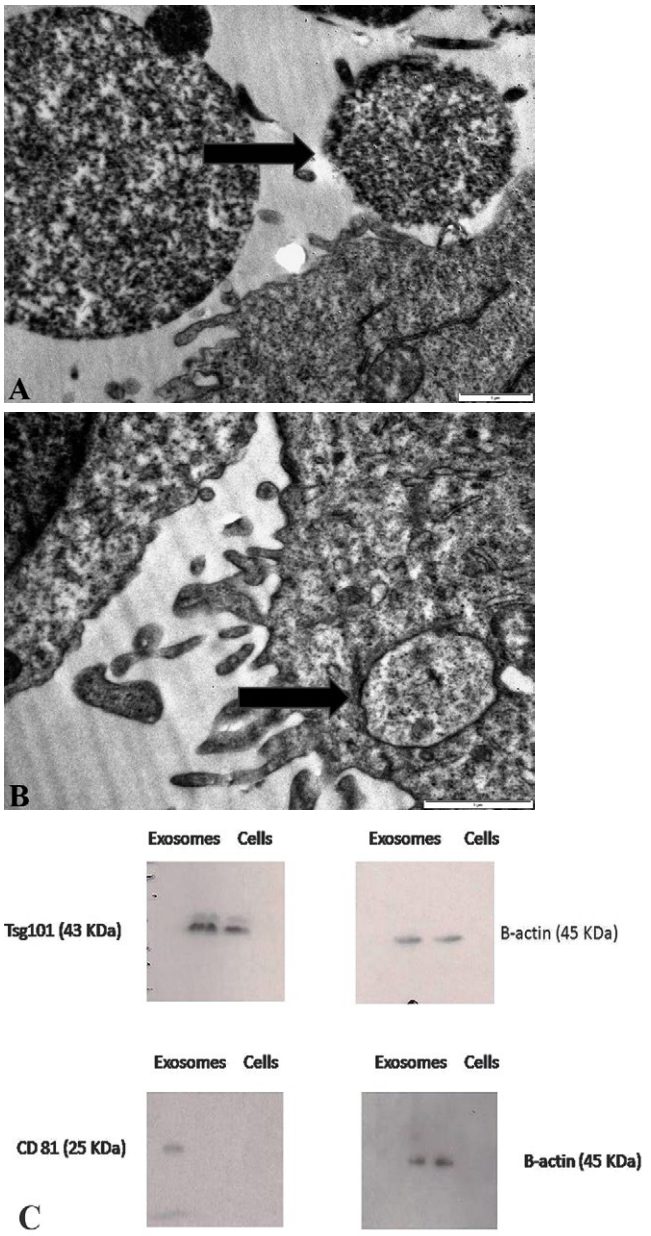

Fig. 2. A, B: Transmision electronmicroscopy images of the exosomes; C: Western blotting characterization of the exosomes (see text).

Cell proliferation potential

The role of miR-375 was assessed in vitro by the cell counting assay. In Fig. 3 we can see the results regarding the growth of the malignant cell population at days 1, 2, 4, 7, 10 and 14. The results of interest are for cells transfected with the miR-375 mimic, where we can clearly see that this small non-coding RNA species inhibits the proliferation of the cancer cells. The cells transfected with the non-specific mimic (NSM) as the negative control represent the natural evolution of the malignancy, whereas the cells transfected with the miR-375 inhibitor represent the positive control. The last control group proves that inhibiting miR-375 has little effect in comparison with its up-regulation. Table I shows the statistical analysis for the HCT116 NSM vs HCT116 miR-375 mimic. Table II shows the statistical analysis for HCT116 NSM vs HCT116 miR-375 inhibitor. As shown in Tables I and II, the microRNA mimic influences cell proliferation at days 7 to 14, whereas the inhibitor only at days 7 and 10. At day 14, the difference between the NSM and the miR-375 inhibitor does not exist anymore, but further studies on other CCR cell lines are needed in order to conclude whether this data reflects what happens in the cancer patient.

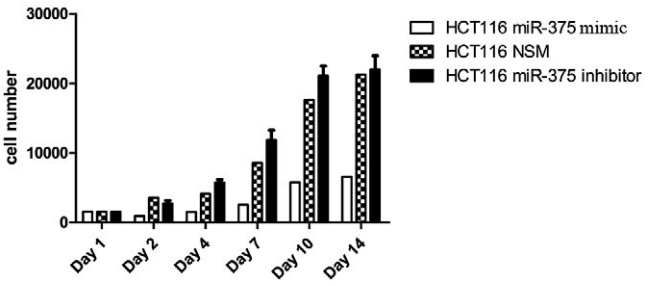

Fig. 3. Cell proliferation assay for the *in vitro* experimental cancer cell growth.

Cell invasion potential

Figure 4 confirms the cell proliferation assay. Thus, cells transfected with the miR mimic have a decreased invasion potential in comparison with the control (NSM). The upper images are those taken from the bottom of the invasion chamber, in white light microscopy. The bottom

Table I. Statistical analysis for the HCT116 NSM vs HCT116 miR-375 mimic

Treatment	HCT116 NSM	HCT116 miR-375 mimic	Difference	95% CI of difference
Day 1	1500	1500	0	-2022 to 2022
Day 2	3533	924,3	-2609	-4631 to -586.6
Day 4	4107	1471	-2636	-4658 to -613.6
Day 7	8567	2500	-6067	-8089 to -4044
Day 10	17600	5767	-11830	-13860 to -9811
Day 14	21280	6557	-14730	-16750 to -12700

Treatment	Difference	t	P value	Summary
Day 1	0	0	P > 0.05	ns
Day 2	-2609	3.947	P<0.01	ns
Day 4	-2636	3.988	P<0.01	ns
Day 7	-6067	9.179	P<0.001	***
Day 10	-11830	17.9	P<0.001	***
Day 14	-14730	22.28	P<0.001	***

***statistical significant difference; ns: not significant statistical difference

Table II. Statistical analysis for HCT116 NSM vs HCT116 miR-375 inhibitor

Treatment	HCT116 NSM	HCT116 miR-375 inhibitor	Difference	95% CI of difference
Day 1	1500	1500	0	-2022 to 2022
Day 2	3533	2700	-833.3	-2856 to 1189
Day 4	4107	5733	1627	-395.8 to 3649
Day 7	8567	11870	3300	1278 to 5322
Day 10	17600	21100	3500	1478 to 5522
Day 14	21280	21960	678	-1344 to 2700

Treatment	Difference	t	P value	Summary
Day 1	0	0	P > 0.05	ns
Day 2	-833.3	1.261	P > 0.05	ns
Day 4	1627	2.461	P > 0.05	ns
Day 7	3300	4.993	P<0.001	***
Day 10	3500	5.295	P<0.001	***
Day 14	678	1.026	P > 0.05	ns

***statistical significant difference; ns: not significant statistical difference

images, show the cells stained with 0.1% Crystal violet. The differences between the NSM (negative control) and the miR inhibitor are not obvious, in correlation with the data from the cell proliferation assay. But when comparing either of the two controls with the miR mimic, one can clearly see the difference in cell number. Thus we can conclude that microRNA-375 inhibits both the proliferation and the invasion potential of CRC.

Apoptosis by flow cytometry

The figures 5A, B and C show the percentage of cells undergoing apoptosis, data obtained by flow cytometry.

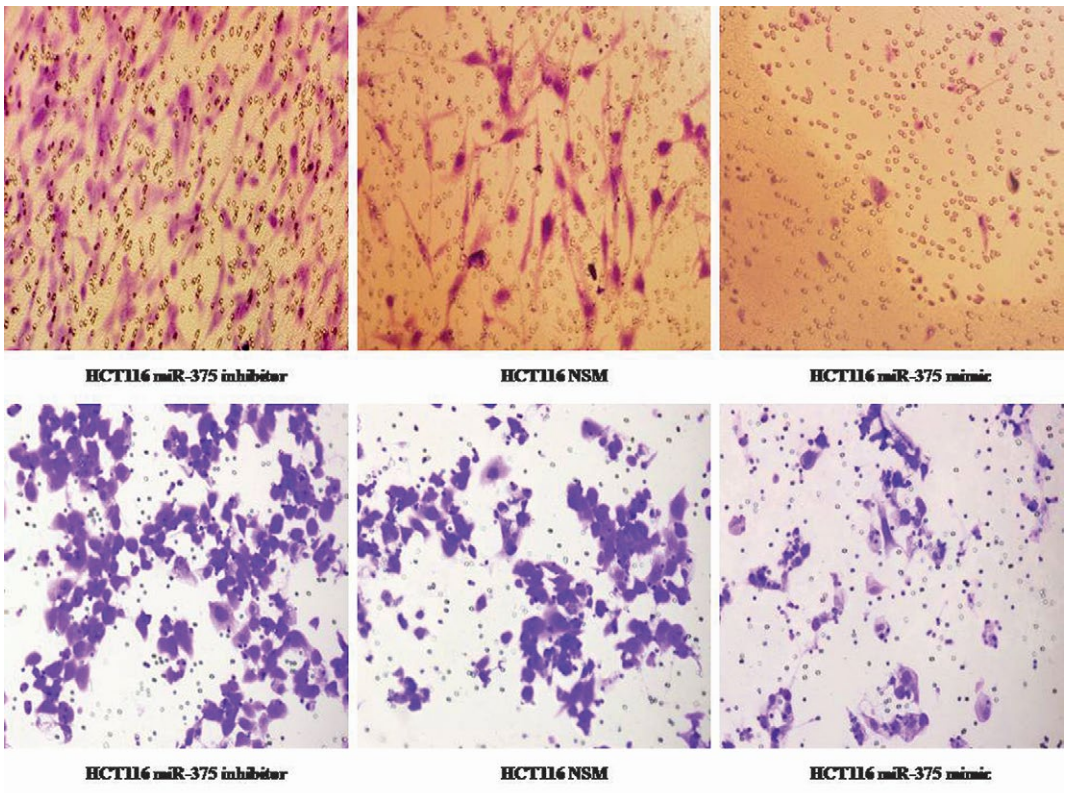

Fig. 4. *In vitro* Matrigel invasion assay for the cancer cells (see text).

Figure 5A shows that 3.84% of the cells transfected with the miR-375 inhibitor are entering apoptosis, image 5B that those transfected with the NSM 6.45% are in apoptosis, whereas image 5C that of those transfected with the mimic, 11.46% are in apoptosis. The data was obtained by flow cytometry.

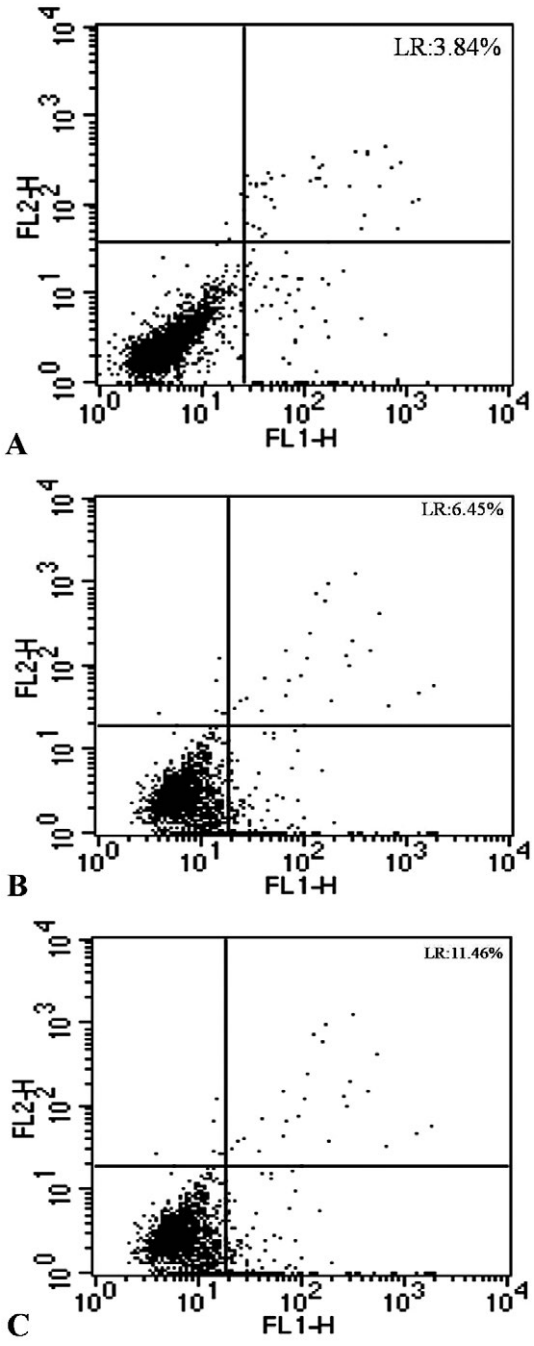

Fig. 5. A, B, C: Percentages of cells undergoing apoptosis, by flow cytometry analysis.

RT-PCR and Western blotting for Bcl-2

RT-PCR for Bcl-2 expression showed that Bcl-2 is down-regulated for miR-375 inhibitor and up-regulated for the miR-375 mimic (Fig. 6). Western blotting was used to investigate whether the gene expression level correlates with the protein levels of Bcl-2. Figure 7 shows that in the case of transfection

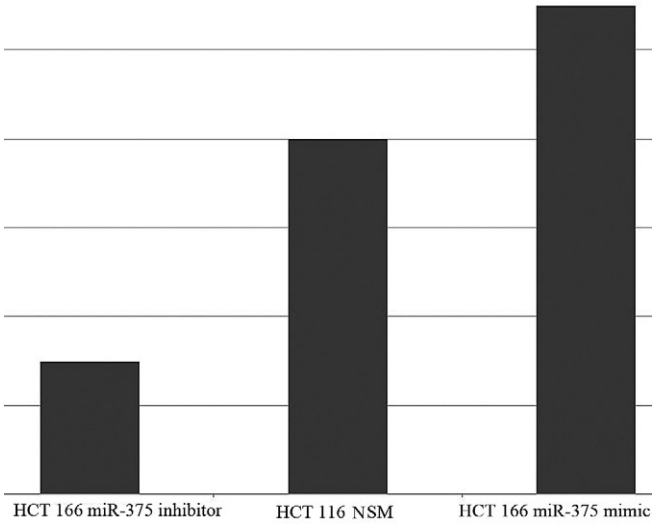

Fig. 6. RT-PCR for Bcl-2 expression.

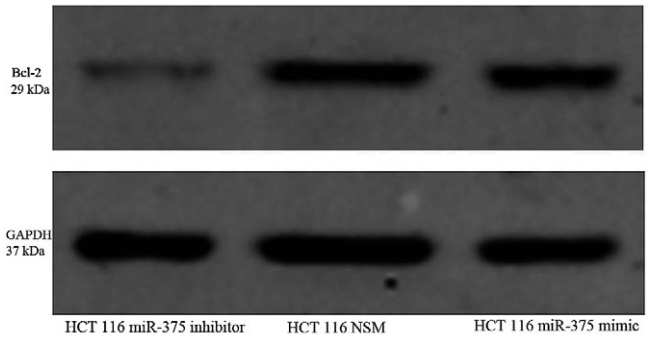

Fig. 7. Western blotting for Bcl-2 expression.

with the microRNA inhibitor, the level of the Bcl-2 protein is decreased, whereas when transfected with the microRNA mimic the protein is secreted in a higher concentration by the CRC cell. The upper images show the protein levels, at 29 kDa and the lower images represent the internal control for the experiment (GAPDH, at 37 kD.

DISCUSSION

MicroRNA-375 was first described as a specific murine pancreatic islet β-cell line MIN6 micro-RNA, with its gene located in human chromosome 2q35 region, a region commonly conserved both for human and mice [35]. After its first description and characterization, the miR-375 role was attributed to pancreatic islet development, glucose homeostasis, mucosa-mediated-immunity, lung surfactant production and secretion, and lately to oncogenesis [36]. In the latter aspect, recent studies revealed that miR-375 is profoundly aberrant (mostly down-regulated) in many human cancers, providing potential prognostic values [37-42] such as head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC), melanoma, glioblastoma multiforme, hepatocellular carcinoma, esophageal cancer, gastric cancer, breast cancer or prostate cancer and it acts by inhibiting/repressing many well-known oncogenes [43, 44]. To this day, it is unclear whether an under-expression of miR-375 offers a poor prognosis [39,

44], or whether its role for prognosis is insignificant or if its expression is not related to cancer outcome [45-48]. miR-375 usage as a potential biomarker in cancer is still unclear and should furthermore be investigated via meta-analyses or prospective multicenter studies with a larger sample size, even if low-expressions have been correlated with poor survival [49]. To exemplify its role in oncogenesis, a restored function of miR-375 in hepatocellular carcinoma inhibited tumor proliferation, invasion potential and migration, and facilitated apoptosis [36]. For the case of gastric cancer, miR-375 was often found to be downregulated and acting as a tumoral suppressor, by inhibiting cellular proliferation via inhibition of the Janus kinase 2 pathway and by facilitating apoptosis via 3-phosphoinositide dependent protein kinase-1 (PDK1) and tyrosine 3-Monooxygenase/ Tryptophan 5-Monooxygenase Activation Protein, Zeta (YHWAZ) [37, 50]. Subsequently, the role of miR-375 in inhibiting proliferation, invasion and metastatic capacity, not only *in vitro*, but also *in vivo*, by the PDK1 pathway, was also found in esophageal squamous cell carcinoma [51, 52]. On the other hand, in prostate cancer, miR-375 was found to be upregulated not only in primary tumor samples but also in the serum, with its role still undetermined [53, 54]. Upregulation of miR-375 was also found in estrogen receptor alpha-(ERα) positive breast cancer cell lines, where its role was considered to be related to cell proliferation [42] leading to low overall survival (OS) and metastatic disease. Furthermore, in invasive lobular breast cancer, mir-375 was suspected to promote progression of disease [55]. Thus, the question arises: does miR-375 act as an oncogene or as a tumor suppressor factor?

In CRC, only recent and few studies have tried to identify the role played by miR-375. In this direction, miR-375 has been found to be significantly under-expressed, without any correlation to tumor size, histological grade, pT stage, pN stage and pTNM stage [56, 57], and that *in vitro* and *in vivo* experiments proved that overexpression of miR-375 may facilitate a direct and targeted growth inhibition for CRC cells either by targeting PI3K/Akt signaling pathway or by stimulating apoptosis via targeting BIRC5 and BCL2L1 [58].

In our study, CRC miR-375 mimic transfected cells showed an inhibition of their proliferation, with results only noticeable after multiple cell cycles, starting from day 7 up until day 10-14 of replication, results previously obtained for miR-365 in a large cohort of samples [59]. Still, even if our study brought positive results, further investigation should be pursued. In the same manner, the invasion potential of CRC miR-375 mimic transfected cells was decreased, even if not as pronounced as the results of the proliferation assay, data seen for the same miR in gastric cancer [60], but when evaluating the apoptotic level, the percentage of miR-375 mimic transfected cells that entered apoptosis was almost double in comparison to the negative control group.

In the current study, once we have established the inhibitory effect of miR-375 on the proliferation and invasion of CCR, the next step was to establish the basic mechanisms of action. The tumor suppressor role of Bcl-2 was first proven at the Johns Hopkins University School of Medicine by the team of Vogelstein [61]: its role in CCR diagnosis and prognosis was further investigated [62, 63], with the loss of Bcl-2 expression being correlated with a higher risk for recurrence in stage II colon adenocarcinoma. As Bcl-2 has been proved to be correlated with cancer progression and dissemination for this malignancy, we further investigated whether miR-375 would be linked to Bcl-2 expression. Thus, cells transfected with either the miR mimic inhibitor or the NSM were investigated at the gene level, as well as the protein level regarding Bcl-2. Our results concluded that in miR-375 positive cells, when the protein can be truly found in higher concentrations, Bcl-2 has indeed a tumor suppressor effect and its inhibition may lead to an increased potential of the cancer to proliferate and invade the surrounding tissues.

Studies have reported that mir-375 is frequently down-regulated in many cancers including esophageal cancer, hepatocellular carcinoma, breast cancer or leukemias. Restoration of mir-375 expression in these cancer cells might inhibit their proliferation by targeting various oncogenes. Cancer cell progression and dissemination has been linked to a reduced susceptibility to drug-induced apoptosis, which was shown to be a consequence, at least in some cases, of over-expression of anti-apoptotic proteins, such as BCL2, IAPs, and BCL-XL [64]. Since mir-375 enhanced the growth inhibitory effect of CRC, we hypothesized that mir-375 might play a role in the modulation of apoptosis. So far, no data that links microRNA-375 to Bcl-2 in cancer has been published, except for the paper of Miao et al. [64].The oncologists from Nanjing have proven that miR-375 was down-regulated in response to *H. pylori* infection in gastric epithelial cell lines. Janus kinase 2 (JAK2) is a well-known target of miR-375, as well as a further activated signal transducer and activator of transcription 3 (STAT3). Both gain-of-function and loss-of-function experiments showed that decreased miR-375 expression could mimic the oncogenic effects of the JAK2–STAT3 pathway. In addition, pretreatment with silent interfering RNAs targeting JAK2 prevented gastric epithelial cells from increasing proliferation and migration even in response to *H. pylori* infection. The JAK2–STAT3 pathway regulated by miR-375 is involved in H. pylori-induced inflammation. This signalling pathway promotes malignant transformation by affecting the expression of BCL-2 and TWIST1. Thus, it offers a potential therapeutic target for inflammation-related cancers, including colon adenocarcinoma.

There are some limitations of this study. It should have included a larger cohort, followed by a wider prospective/ multicentric clinical study. Since miR-375 has been found to have an up-regulated rectal-cancer-specific molecular profile [15-18], the findings in this present study could raise awareness upon the fact that this specific miR is also present in colon cancer, and that the outcomes regarding proliferation, increased invasion and metastatic capacity, will only rely upon its expression (over-/under-expression). Also, being mainly an *in vitro* study performed in a laboratory setting, the molecular profile and the *in vivo* repercussions could be altered.

CONCLUSION

To conclude, the novelty of this present study is that it brings to the forefront new daunting hypotheses according to which miR-375, as previously mentioned in other studies, plays

an important role in controlling the pathways responsible for inhibiting the natural history of CRC tumor cells, via the Bcl-2 pathway. Still, future studies should include *in vivo* work-up and should gather prospective data in order to move forward and initiate a clinical prospective study on a large population cohort.

Conflicts of interest. The authors report no potential conflict of interest.

Authors' contribution. F.Z., M.S.M., B.P., C.B., G.G., S.S., A.J., R.C.P., C.I.L., V.P., L.P., D.E., R.Z., M.A.M. and C.T. performed the experiments. M.S.M., I.B.N., F.Z. and C.T. wrote the manuscript. All authors read the final version of the manuscript and approved the submission.

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REFERENCES

1. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics. CA Cancer J Clin 2014; 64: 104–117. doi: 10.3322/caac.21220
2. Lujambio A, Lowe SW. The microcosmos of cancer. Nature 2012; 482: 347-355. doi: 10.1038/nature10888
3. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105: 10513-10518. doi: 10.1073/pnas.0804549105
4. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866. doi: 10.1038/nrc1997
5. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with rognosis and therapeutic outcome in colon adenocarcinoma. JAMA 2008; 299: 425-436. doi: 10.1001/jama.299.4.425
6. Wittmann J, Jack HM. Serum microRNAs as powerful cancer biomarkers. Biochim Biophys Acta 2010; 1806: 200-207. doi: 10.1016/j.bbcan.2010.07.002
7. Gaedcke J, Grade M, Camps J, et al. The rectal cancer microRNAome--microRNA expression in rectal cancer and matched normal mucosa. Clin Cancer Res 2012; 18: 4919-4930. doi: 10.1158/1078-0432.CCR-12-0016
8. Pucci S, Mazzarelli P. MicroRNA dysregulation in colon cancer microenvironment interactions: the Importance of small things in metastases. Cancer Microenviron 2011; 4: 155-162. doi: 10.1007/s12307-011-0062-y
9. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Res 2008; 36: D154-D158. doi: 10.1093/nar/gkm952
10. Gaedcke J, Grade M, Jung K, et al. Mutated KRAS results in overexpression of DUSP4, a MAP-kinase phosphatase, and SMYD3, a histone methyltransferase, in rectal carcinomas. Genes Chromosomes Cancer 2010; 49: 1024-1034. doi: 10.1002/gcc.20811
11. Chang KH, Mestdagh P, Vandesompele J, Kerin MJ, Miller N. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. BMC Cancer 2010; 10: 173. doi: 10.1186/1471-2407-10-173
12. Zhang H, Li Y, Huang Q, et al. MiR-148a promotes apoptosis by targeting Bcl-2 in colorectal cancer. Cell Death Differ 2011; 18: 1702–1710. doi: 10.1038/cdd.2011.28
13. Zhang Y, Li M, Wang H, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. World J Surg 2009; 33: 698–709. doi: 10.1007/s00268-008-9833-0
14. Babashah S, Soleimani M. The oncogenic and tumour suppressive roles of microRNAs in cancer and apoptosis. Eur J Cancer 2011; 47: 1127–1137. doi: 10.1016/j.ejca.2011.02.008
15. Xu L, Li M, Wang M, Yan D, Feng G, An G. The expression of microRNA-375 in plasma and tissue is matched in human colorectal cancer. BMC Cancer 2014; 14: 714. doi: 10.1186/1471-2407-14-714
16. Zhang JX, Song W, Chen ZH, et al. Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis. Lancet Oncol 2013; 14: 1295-1306. doi: 10.1016/S1470-2045(13)70491-1
17. Wang CJ, Zhou ZG, Wang L, et al. Clinicopathological significance of microRNA-31, −143 and −145 expression in colorectal cancer. Dis Markers 2009; 26: 27-34. doi: 10.3233/DMA-2009-0601
18. Schee K, Boye K, Abrahamsen TW, Fodstad O, Flatmark K. Clinical relevance of microRNA miR-21, miR-31, miR-92a, miR-101, miR-106a and miR-145 in colorectal cancer. BMC Cancer 2012; 12: 505. doi: 10.1186/1471-2407-12-505
19. Elzagheid A, Buhmeida A, Korkeila E, Collan Y, Syrjanen K, Pyrhonen S. Nuclear beta-catenin expression as a prognostic factor in advanced colorectal carcinoma. World J Gastroenterol 2008; 14: 3866–3871. doi: 10.3748/wjg.14.3866
20. Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 2004; 432: 226–230. doi: 10.1038/nature03076
21. Zhou J, Song S, He S, et al. MicroRNA-375 targets PDK1 in pancreatic carcinoma and suppresses cell growth through the Akt signaling pathway. Int J Mol Med 2014; 33: 950–956. doi: 10.3892/ijmm.2014.1638
22. Faltejskova P, Svoboda M, Srutova K, et al. Identification and functional screening of microRNAs highly deregulated in colorectal cancer. J Cell Mol Med 2012; 16: 2655–2666. doi: 10.1111/j.1582-4934.2012.01579.x
23. Dai X, Chiang Y, Wang Z, et al. Expression levels of microRNA-375 in colorectal carcinoma. Mol Med Rep 2012; 5: 1299–1304. doi: 10.3892/mmr.2012.815
24. Wang Y, Tang Q, Li M, Jiang S, Wang X. MicroRNA-375 inhibits colorectal cancer growth by targeting PIK3CA. Biochem Biophys Res Commun 2014; 444: 199–204. doi: 10.1016/j.bbrc.2014.01.028
25. Christensen LL, Holm A, Rantala J, et al. Functional screening identifies miRNAs influencing apoptosis and proliferation in colorectal cancer. PLoS One 2014; 9: e96767. doi: 10.1371/journal.pone.0096767
26. Yan JW, Lin JS, He XX. The emerging role of miR-375 in cancer. Int J Cancer. 2014; 135: 1011-1018. doi: 10.1002/ijc.28563
27. Dai X, Chiang Y, Wang Z, et al. Expression levels of microRNA-375 in colorectal carcinoma. Mol Med Rep 2012; 5: 1299-1304. doi: 10.3892/mmr.2012.815
28. Li L, Masica D, Ishida M, et al. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. Hepatology 2014; 60: 896-907. doi: 10.1002/hep.27050
29. Braicu C, Tomuleasa C, Monroig P, Cucuianu A, Berindan-Neagoe I, Calin GA. Exosomes as divine messengers: are they the Hermes of

modern molecular oncology? Cell Death Differ 2015; 22: 34-45. doi: 10.1038/cdd.2014.130
30. Tomuleasa C, Soritau O, Orza A, et al. Gold nanoparticles conjugated with cisplatin/doxorubicin/capecitabine lower the chemoresistance of hepatocellular carcinoma-derived cancer cells. J Gastrointestin Liver Dis 2012; 21: 187-196.
31. Orza A, Soriţău O, Tomuleasa C, et al. Reversing chemoresistance of malignant glioma stem cells using gold nanoparticles. Int J Nanomedicine 2013; 8: 689-702. doi: 10.2147/IJN.S37481
32. Petrushev B, Tomuleasa C, Soritau O, et al. Metformin plus PIAF combination chemotherapy for hepatocellular carcinoma. Exp Oncol 2012; 34: 17-24.
33. Miklášová N, Fischer-Fodor E, Lönnecke P, et al. Antiproliferative effect of novel platinum(II) and palladium(II) complexes on hepatic tumor stem cells in vitro. Eur J Med Chem 2012; 49: 41-7. doi: 10.1016/j.ejmech.2011.12.001
34. Peng H, Ishida M, Li L, et al. Pseudogene INTS6P1 regulates its cognate gene INTS6 through competitive binding of miR-17-5p in hepatocellular carcinoma. Oncotarget 2015; 6: 5666-5677.
35. Baroukh NN, Van Obberghen E. Function of microRNA-375 and microRNA-124a in pancreas and brain. FEBS J 2009; 276: 6509-6521. doi: 10.1111/j.1742-4658.2009.07353.x
36. Yan JW, Lin JS, He XX. The emerging role of miR-375 in cancer. Int J Cancer 2014; 135: 1011-1018. doi: 10.1002/ijc.28563
37. He XX, Chang Y, Meng FY, et al. MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth in vitro and in vivo. Oncogene 2012; 31: 3357-3369. doi: 10.1038/onc.2011.500
38. Ding L, Xu Y, Zhang W, et al. MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. Cell Res 2010; 20: 784-793. doi: 10.1038/cr.2010.79
39. Mathé EA, Nguyen GH, Bowman ED, et al. MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. Clin Cancer Res 2009; 15: 6192-6200. doi: 10.1158/1078-0432.CCR-09-1467
40. Avissar M, Christensen BC, Kelsey KT, Marsit CJ. MicroRNA expression ratio is predictive of head and neck squamous cell carcinoma. Clin Cancer Res 2009; 15: 2850-2855. doi: 10.1158/1078-0432.CCR-08-3131
41. Szczyrba J, Nolte E, Wach S, et al. Downregulation of Sec23A protein by miRNA-375 in prostate carcinoma. Mol Cancer Res 2011; 9: 791-800. doi: 10.1158/1541-7786.MCR-10-0573
42. de Souza Rocha Simonini P, Breiling A, Gupta N, et al. Epigenetically deregulated microRNA-375 is involved in a positive feedback loop with estrogen receptor alpha in breast cancer cells. Cancer Res 2010; 70: 9175-9184. doi: 10.1158/0008-5472.CAN-10-1318
43. Yu H, Jiang L, Sun C, et al. Decreased circulating miR-375: a potential biomarker for patients with non-small-cell lung cancer. Gene 2014; 534: 60-65. doi: 10.1016/j.gene.2013.10.024
44. Harris T, Jimenez L, Kawachi N, et al. Low-level expression of miR-375 correlates with poor outcome and metastasis while altering the invasiveproperties of head and neck squamous cell carcinomas. Am J Pathol 2012; 180: 917-928. doi: 10.1016/j.ajpath.2011.12.004
45. Jonsdottir K, Janssen SR, Da Rosa FC, et al. Validation of expression patterns for nine miRNAs in 204 lymph-node negative breast cancers. PLoS One 2012; 7: e48692. doi: 10.1371/journal.pone.0048692
46. Carlsen AL, Joergensen MT, Knudsen S, de Muckadell OB, Heegaard NH. Cell-free plasma microRNA in pancreatic ductal adenocarcinoma and disease controls. Pancreas 2013; 42: 1107-1113. doi: 10.1097/MPA.0b013e318296bb34
47. Bryant RJ, Pawlowski T, Catto JW, et al. Changes in circulating microRNA levels associated with prostate cancer. Br J Cancer 2012; 106: 768-774. doi: 10.1038/bjc.2011.595
48. Stroup DF, Berlin JA, Morton SC, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. JAMA 2000; 283: 2008-2012. doi: 10.1001/jama.283.15.2008
49. Shao Y, Geng Y, Gu W, Huang J, Ning Z, Pei H. Prognostic significance of microRNA-375 downregulation in solid tumors: a meta-analysis. Dis Markers 2014; 2014: 626185. doi: 10.1155/2014/626185
50. Tsukamoto Y, Nakada C, Noguchi T, et al. MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. Cancer Res. 2010; 70: 2339-2349. doi: 10.1158/0008-5472.CAN-09-2777
51. Kong KL, Kwong DL, Chan TH, et al. MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma throughrepressing insulin-like growth factor 1 receptor. Gut 2012; 61: 33-42. doi: 10.1136/gutjnl-2011-300178
52. Li X, Lin R, Li J. Epigenetic silencing of microRNA-375 regulates PDK1 expression in esophageal cancer. Dig Dis Sci 2011; 56: 2849-2856. doi: 10.1007/s10620-011-1711-1
53. Wach S, Nolte E, Szczyrba J, et al. MicroRNA profiles of prostate carcinoma detected by multiplatform microRNA screening. Int J Cancer 2012; 130: 611-621. doi: 10.1002/ijc.26064
54. Selth LA, Townley S, Gillis JL, et al. Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease. Int J Cancer 2012; 131: 652-661. doi: 10.1002/ijc.26405
55. Giricz O, Reynolds PA, Ramnauth A, et al. Hsa-miR-375 is differentially expressed during breast lobular neoplasia and promotes loss of mammary acinar polarity. J Pathol 2012; 226: 108-119. doi: 10.1002/path.2978
56. Dai X, Chiang Y, Wang Z, et al. Expression levels of microRNA-375 in colorectal carcinoma. Mol Med Rep 2012; 5: 1299-1304. doi: 10.3892/mmr.2012.815
57. Wang Y, Tang Q, Li M, Jiang S, Wang X. MicroRNA-375 inhibits colorectal cancer growth by targeting PIK3CA. Biochem Biophys Res Commun 2014; 444: 199-204. doi: 10.1016/j.bbrc.2014.01.028
58. Christensen LL, Holm A, Rantala J, et al. Functional screening identifies miRNAs influencing apoptosis and proliferation in colorectal cancer. PLoS One 2014; 9: e96767. doi: 10.1371/journal.pone.0096767
59. Nie J, Liu L, Zheng W, et al. microRNA-365, down-regulated in colon cancer, inhibits cell cycle progression and promotes apoptosis of colon cancer cells by probably targeting Cyclin D1 and Bcl-2. Carcinogenesis 2012; 33: 220-225. doi: 10.1093/carcin/bgr245
60. Shen ZY, Zhang ZZ, Liu H, Zhao EH, Cao H. miR-375 inhibits the proliferation of gastric cancer cells by repressing ERBB2 expression. Exp Ther Med 2014; 7: 1757-1761. doi: 10.3892/etm.2014.1627
61. Pietenpol JA, Papadopoulos N, Markowitz S, Willson JK, Kinzler KW, Vogelstein B. Paradoxical inhibition of solid tumor cell growth by bcl2. Cancer Res 1994; 54: 3714-3717.
62. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. Proc Natl Acad Sci U S A 2003; 100: 1931-1936. doi: 10.1073/pnas.2627984100
63. Poincloux L, Durando X, Seitz JF, et al. Loss of Bcl-2 expression in colon cancer: a prognostic factor for recurrence in stage II colon cancer. Surg Oncol 2009; 18: 357-365. doi: 10.1016/j.suronc.2008.09.003
64. Miao L, Liu K, Xie M, Xing Y, Xi T. miR-375 inhibits Helicobacter pylori-induced gastric carcinogenesis by blocking JAK2-STAT3 signaling. Cancer Immunol Immunother 2014; 63: 699-711. doi: 10.1007/s00262-014-1550-y