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 CRC. 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.

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 colon-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 pathways, such as MAPK, Wnt, TGF-beta signal pathways 375 targets genes that play an important role in regulating tumorigenesis was previously described [19-21]. 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 acetone of increasing concentrations (between 50–100%, 15 minutes each), infiltration with solutions of Epon 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 aceton (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 3600x and 19 000x. 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).
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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 50μl of the mixture. Afterwards, the Matrigel was allowed to solidify at 37°C for 4h. After solidification, 5 × 10^5 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^(-ΔΔCt). 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^-ΔCt*.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
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 endoplasmatic 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.

**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.

**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
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).](image)
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.

RT-PCR and Western blotting for Bcl-2

RT-PCR for Bcl-2 expression showed that Bcl-2 is downregulated 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 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,
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 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.

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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.D., E.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


