

MiR-708-5p as a Predictive Marker of Colorectal Cancer Prognosis

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Abstract: *Background:* MicroRNAs (miRNA) are short non-coding RNA that act as negative regulators of gene expression. Altered levels of miR-708-5p have recently been described in many tumors, although its contribution in colorectal cancer (CRC) pathophysiology remains unclear.

Methods/Patients: Quantitative real-time polymerase chain reaction was employed to evaluate the expression of miR-708-5p in 50 CRC and 20 paired adjacent noncancerous tissues. The relationship between miRNA levels and clinicopathological features was estimated using the Mann-Whitney test, and survival curves calculated by the Kaplan-Meier method. Additionally, *in vitro* assays were performed to investigate the possible role of miR-708-5p on CRC cell survival.

Results: The expression level of miR-708-5p was significantly decreased in CRC tissues (3.79 fold-change, $p=0.0112$) when compared with non-neoplastic colon samples. Paired analysis in 20 CRC samples with their corresponding adjacent non-neoplastic tissue showed miR-708 downregulation in 60% of them. The same pattern was seen in DLD1 and HT-29 cell lines (~50-fold decrease). Interestingly, higher expression is observed in patients with poor prognosis such as stage III/IV, relapse/metastasis and death, and shorter 5-year event free survival. Exogenous expression of miR-708 exerted a significant influence on clonogenicity *in vitro*.

Conclusion: These results suggest that reduced miR-708-5p expression may contribute to the first stages of colorectal carcinogenesis. A shift in the regulation of miR-708-5p might operate in more severe stages of the disease. It seems that lower levels of miR-708 expression might connote less advanced disease and better prognosis. Further studies are needed to corroborate our results and better elucidate the role of miR-708 in CRC.

Keywords: microRNA, colorectal tumor, DLD1, HT-29, cell lines.

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy worldwide and its incidence and mortality have significantly increased in the past decades [1-3]. Despite improvements in surgery and adjuvant chemotherapy, 5-year survival rates vary significantly with tumor progression, and CRC persists as one of the leading causes of cancer-related death worldwide [2-4]. Therefore, the identification of more sensitive and specific biomarkers that can accurately identify biological characteristics of tumors and predict prognosis, along with further recognition of new potential targets for therapy, are urgently needed.

In this context, abnormal microRNA (miRNA) expression has been suggested to be a key contributor to carcinogenesis, participating in the onset and/or progression of several human tumors. Moreover, miRNA expression profiling has identified patterns able to distinguish tumor from normal tissue, predict disease outcome and evaluate aggressiveness in several tumor types such as acute lymphoblastic leukemia, breast, colorectal, hepatic, lung, and pancreatic cancer, among others [1, 5-7].

MiRNAs are small (17-25 nucleotides), single-stranded non-coding RNAs that function predominantly as negative regulators of gene expression by targeting mRNA. Currently, almost 2,000 miRNAs have been described in the human genome and, although most of them still have not been related with any specific function, the association between disruption of their expression and tumor aggressiveness is widely

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accepted [8-9]. One emerging miRNA pointed as a prognostic tool for some tumor types is miR-708-5p. Its differential expression has recently been associated with numerous tumor types, including leukemia, lymphoma, glioblastoma, lung cancer, and bladder and laryngeal squamous cell carcinomas [6-7, 10-15]. *In vitro*, forced expression of miR-708-5p was able to induce apoptosis and suppress tumorigenicity in renal, and prostate tumors *via* regulation of Survivin [16], CD44 and AKT2 [17], suggesting an important role for miR-708-5p in cancer progression.

The contribution of miR-708-5p deregulation in CRC is still uncertain. Accordingly, the present study aimed to evaluate miR-708-5p expression in CRC samples and to associate its expression levels with clinicopathological and prognostic features. Moreover, the effect of miR-708-5p induced over-expression in clonogenicity, proliferation and apoptosis were also assessed using CRC cell line models.

MATERIAL AND METHODS

Tissue Samples

Surgical specimens of primary CRC were obtained from 50 sequential patients at the Clinics University Hospital (Ribeirao Preto School of Medicine – University of Sao Paulo), between April 2008 and February 2009, the survival analysis was followed until October 2013. Samples diagnosed as inflammatory bowel disease and tumors that were not adenocarcinomas, such as intestinal stromal tumor and squamous cell carcinoma were excluded. Tissue specimens were obtained from a non-necrotic area of the tumor and from autologous normal mucosa from the same patient at a resection margin located at more than 3 cm from the tumor. The 50 patients corresponded to 25 women and 25 men with a mean age at diagnosis of 62.5 years (range: 27–89 years old). Twenty-six samples were localized in the colon (6 in the ascending colon, 2 in the transverse colon), 9 in the sigmoid colon and 13 in the rectum. One sample was classified as stage I, 21 as stage II, 17 as stage III, and 11 patients were classified as IV stage according to TNM (tumors/nodes/metastases) classification. Angiography testing showed 18 negative samples, 18 positive and for 14 there was no available data. The range of carcinoembryonic antigen (CEA) was 0.26 – 182.00 ng/mL (with a mean 4.50 ng/mL). Twenty-one patients presented metastasis (10 to the lung and 11 to the liver) and 4 had local relapse. This study was approved by the local Ethics Committee that follows the

Helsinki convention criteria. Signed statement of informed consent was obtained from each patient.

MiRNA Quantification by qRT-PCR

Frozen tumor tissues samples were macrodissected by a surgeon. Total RNA was extracted using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. The quantity and quality of samples were evaluated with an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). Total RNA (100 ng) was retrotranscribed with miRNA-specific primers using a TaqMan[®] Reverse Transcription Reagents (hsa-miR-708, ID 002341) (Applied Biosystems, Foster City, CA), and qRT-PCR was then performed using Taqman[®] miRNA assays according to the manufacturer's protocol.

The expression level of miR-708-5p was measured using the ABI 7500 Real Time PCR System (PE Applied Biosystems). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [18] with two internal controls, small nuclear RNU6B and RNU48, used to normalize the miRNAs level. The CT's mean expression of a pool of 5 non-neoplastic samples was used as calibrator. qRT-PCR was performed in duplicate and a standard deviation (SD) of < 0.5 between duplicates was accepted. A blank control was run in parallel to determine the absence of contamination within each experiment.

Cells Lines

The CRC cell lines, DLD1 and HT-29, were obtained from the ATCC (American Type Culture Collection, Rockville, MD) and cultured with recommended media supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂.

Transfection of miRNA

Pre-miRNA miR-708-5p and scramble control (Ambion Pre-miR miRNA Precursors[®], AB) were reverse-transfected into CRC cell lines using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 125 nM. The miRNAs transfection efficiency was monitored by qRT-PCR at 24 hours. The transfection agent cytotoxicity was monitored using results from the endogenous control amplification: $CT^{scramble}/CT^{nontransfected}$ = cytotoxicity factor, values close to 1 indicate lower toxicity.

Measurement of Cell Growth

Cell survival was assessed using the resazurin reduction assay (Sigma Aldrich St. Louis, MO, United States). Briefly, 24 hours after transfection, the cells were seeded in 96-well flat-bottom plates (1,000 cells/well) and were then incubated for 24 until 144 hours after transfection. After each period 10 μ L of resazurin (40 mol/l approximately) reagent was added in each well. The plates were incubated for 6 hours at 37°C and read at 570 nm by using an iMark microplate reader (Bio- Rad Laboratories®). Each experiment was performed in quintuplicate wells and repeated in four sets of tests.

Colony Formation Assay

Clonogenic assays were performed according to Franken *et al.* [19]. After trypsinization, single cell suspensions of 300 cells were seeded in 6-well plates after 24 hour of transfection. Cell cultures were incubated for 12 days. Colonies were then rinsed with PBS, fixed with methanol and stained with Giemsa. Only colonies with above 50 cells were counted. Assays were performed in triplicate and repeated in three sets of tests.

Detection of Apoptotic Cells

For apoptosis 20,000 cells were seeded on 12-well plates containing 2 ml of culture medium after 24 hours transfection. After 72 hours, caspase activity was measured through the NucView™ 488 Caspase-3 Detection in Living Cells kit (Biotium Inc. Hayward, CA, USA) according to the manufacturer's instructions. Concisely, transfected cells were trypsinized and incubated for 40 minutes at room temperature with the Caspase-3 substrate. Then cells were fixed in formaldehyde, counterstained with 4', 6-diamidino-2-phenylindole (DAPI), mounted, coverslipped and analyzed by fluorescence microscopy with a triple filter. Five hundred nuclei were analyzed per treatment and cells were scored and categorized according to differential staining. Assays were repeated in three sets of tests.

Statistical Analysis

The association among the following variables: age (< 60 years *versus* \geq 60 years), topography (colon *versus* rectum), disease stage at diagnosis (stage 1 or 2 *versus* stage 3 or 4), vascular invasion (angiography negative *versus* positive), CEA (< 15 ng/mL *versus* \geq 15 ng/mL), metastasis (presence *versus* absence); liver

metastasis (presence *versus* absence); lung metastasis (presence *versus* absence); local relapse (presence *versus* absence), event-free survival (relapse/metastasis *versus* complete remission), overall survival (alive *versus* deceased) and expression levels the miR-708-5p was determined by Mann-Whitney tests. Survival analysis was carried out based on Kaplan-Meier curves, using the median of the miRNA expression observed in control samples as cut-off. The Cox proportional regression model was used for multivariable analysis of prognostic factors and also to estimate the association between the variables and risk of an unfavorable event, with hazard ratio (HR) and 95% confidence interval (95% CI).

The functional assays data were statistically analyzed by Student's two-tailed t-test. All tests were carried out for $\alpha = 0.05$. All data were analyzed using the SPSS 21.0 software (SPSS Inc, IL, USA) and the figures designed using GraphPad Prism 6 (GraphPad Software Inc., CA, USA). The Ingenuity Pathway Analysis (IPA) software (QIAGEN, Redwood, CA) was used to determine which pathways and biological processes are enriched.

RESULTS

MiR-708-5p Expression is Down-Regulated in CRC Samples

Down-regulation of miR-708-5p was observed in 60% of CRC samples compared with their paired adjacent non-neoplastic tissue (n=20), (Figure 1a). Analyzing a larger sample, the expression level of miR-708-5p was significantly decreased in CRC tissues (n=50) compared with non-neoplastic colon samples (n=20; 3.79 fold-change, p=0.0112) (Figure 1b). Interestingly, when evaluating the prognostic potential of miR-708-5p among CRC samples, higher expression levels were associated with poor prognosis. The expression of miR-708-5p during stage I and II was lower when compared to more advanced stages (III and IV) in CRC (2.00 fold-change; p = 0.015) (Figure 2a). Increased expression was also observed in samples from patients who eventually showed remission and relapse/metastasis (1.89 fold-change; p = 0.002) (Figure 2b), and the same pattern was observed comparing samples from alive with deceased patients (3.79 fold-change; p = 0.013) (Figure 2c). Moreover, expression values above the median for miR-708-5p were associated with shorter event free 5-year survival (EFS) (p = 0.005) (Figure 2d). In univariate analysis it was found a significant lower 5

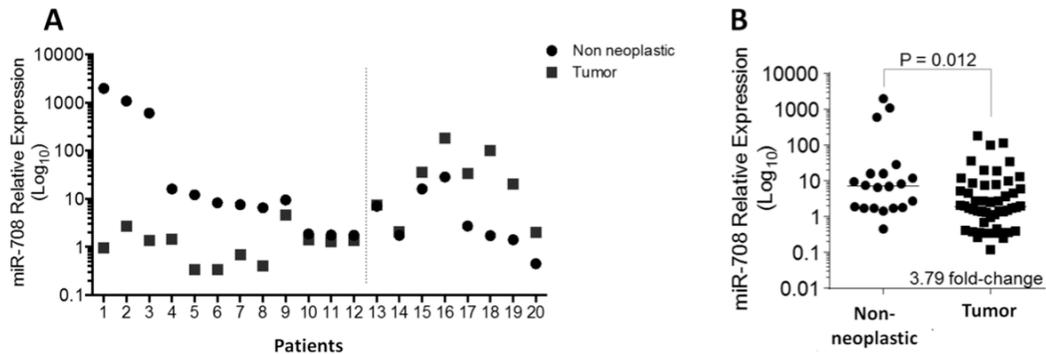


Figure 1: Expression of miR-708-5p was positively associated with CRC. (a) Expression levels in adjacent non-neoplastic samples (n=20; circle) and paired tumors samples (n=20; square); (b) Expression levels in non-neoplastic samples (n=20; right) and non paired tumors samples (n=50; left). The expression levels the miR-708-5p were analyzed by the Wilcoxon and t test to paired data and Mann-Whitney.

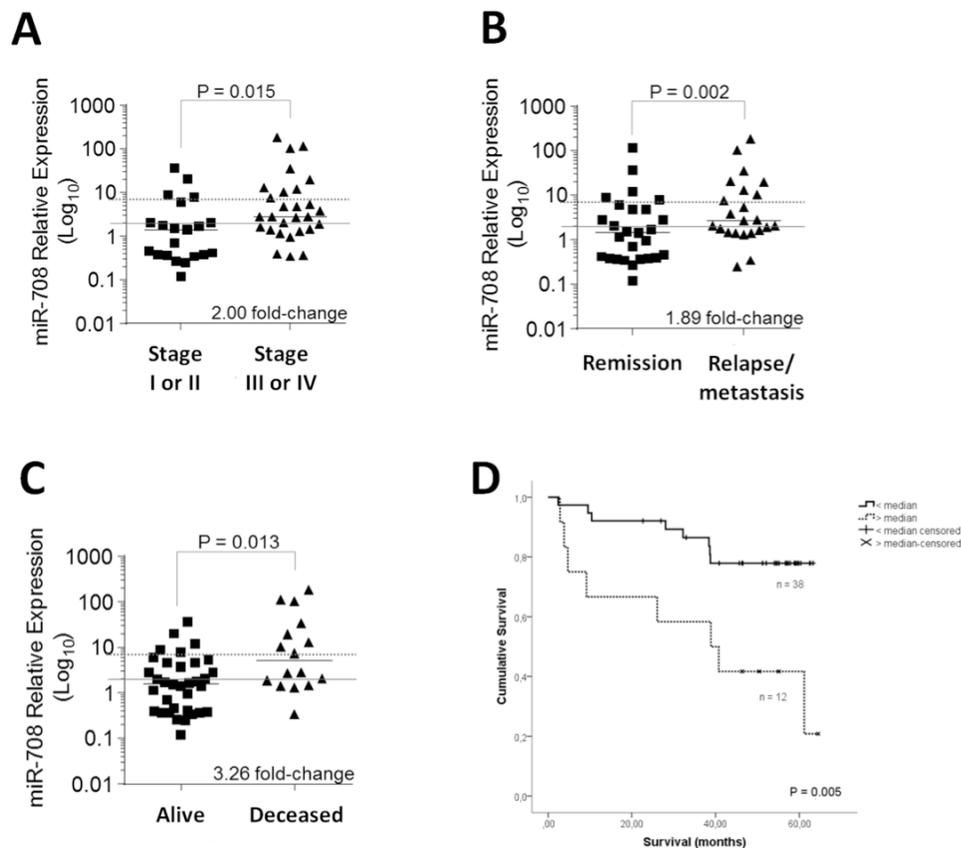


Figure 2: Relative expression levels of miR-708-5p in CRC patients stratified according to biological and clinical features. (a) Comparison between CRC patients in stages 1 or 2 (n=22; right) and 3 or 4 (n=28; left); (b) Expression levels in CRC patients with remission (n=27; right) and relapse or metastasis (n=23; left); (c) Expression in alive (n=34; right) and deceased (n=16; left) CRC patients; (d) OS according to the level of expression of miR-708-5p (below and above median of control tissue expression). The expression levels the miR-708-5p were analyzed by the Mann-Whitney and survival analysis was carried out based on Kaplan-Meier curves. The full black line represents the median of miR-708-5p in each group. Dotted grey lines represent the median of non-neoplastic samples and the full grey line the median of tumors of miR-708-5p expression.

years overall survival to the variables stage III/IV ($P = 0.001$) and mir-708 expression level higher than median of the normal control expression. ($P = 0.005$). No significant differences in overall survival was observed to the variables age ($P = 0.055$), topography

($P = 0.104$), vascular invasion, CEA values ($P = 0.628$) and gender ($P = 0.408$). Multivariate analysis using stage and miR-708 expression show that miR-708 was an independent prognostic factor ($P = 0.035$) (Table 1).

Table 1: Multivariate Analysis Using the Cox Regression Model for the Variables Stage and miR-708 Expression

| | Hazard Ratio | 95% CI | P values |
|--------------------------|--------------|--------------|----------|
| Stage I/II versus III/IV | 6.718 | 1.504-30.009 | 0.013 |
| miR-708 expression | 2.920 | 1.078-7.913 | 0.035 |

95% CI - 95% confidence interval.

These findings suggest that higher miR-708-5p expression levels within CRC patients might represent a potential poor prognosis marker.

MiR-708-5p is Downregulated in CRC Cell Lines and its Exogenous Expression Reduces the Clonogenic Capacity In Vitro

MiR-708-5p was observed downregulated in DLD1 and HT-29 CRC cell lines with 53.7 and 58.3-fold change, respectively, compared to tissue from non-neoplastic mucosa (Figure 3a). When miR-708-5p was introduced through transfection the expression increased around 42 and 20 thousand fold-change in DLD1 and HT-29 CRC cell lines, respectively, compared to scramble control (Figure 3b). Expression levels were maintained until 72h post-transfection. The transfection agent was not toxic with values above 0.94. After miR-708-5p exogenous expression, the clonogenic assay demonstrated a significantly reduction in colony formation capacity for both cell lines, ($p < 0.0001$). Clonogenicity decreased in 71% for DLD1 cells and 56% for the HT-29 cell line (Figure 3c). In parallel, miR-708-5p forced expression showed a tenuous (10%) increase in apoptosis compared with scramble controls after 72 hours in both cell lines, alteration of proliferation was unsubstantial even after 144 hours (data not shown).

DISCUSSION

MiRNAs are associated with the development of normal tissues and their deregulation has been reported in several cancers [20-21]. MiRNA expression patterns are complex and specific to different malignancies [8, 22]. In CRC, there are several descriptions of altered miRNAs, many of which are relevant to cancer development and show potential usefulness as diagnostic and prognostic markers [23-24]. Nonetheless, the contribution of altered miR-708-5p levels remains unclear.

So far, two articles have described miR-708-5p up-regulation in CRC [25-26]. However, in both, the number of tumor samples was reduced (n between 5 and 19) with limited clinical data. In opposition, we

found a significantly lower expression of miR-708-5p in CRC samples compared with normal mucosa. In fact, when tumor samples were paired with the corresponding adjacent non-neoplastic tissue, 60% of samples showed downregulation of miR-708-5p. In accordance with our results, downregulation of this miRNA has also been demonstrated in different cancer types including glioblastoma [10]; T-cell acute lymphoblastic leukemia [27]; renal cell carcinoma [16] and prostate cancer [17]. Conversely, overexpression was also found in other malignancies, such in B-cell acute lymphoblastic leukemia [12], bladder carcinoma [15] and laryngeal squamous cell carcinoma [6].

As most miRNAs, miR-708-5p can target multiple mRNAs and regulate gene expression with antagonist functions. Some of its targets like AKT1, CCND1, EZH2, PARP-1, BCL2 [10]; AKT2 [16]; Survivin, ZEB2, BMI1 [17], CDKN2B [25], show oncogenic potential, while others, such as Caspase-2 [15] and NNAT [12] show tumor suppressor activity. Consequently, the diversity of targets may explain how a unique miRNA could play counteracting roles in the pathogenesis of different types of cancer according to the mRNAs profile available in each cell type or moment.

Enrichment analysis performed by Ingenuity Pathway Analysis -IPA- (Qiagen, Redwood, CA, USA) showed 876 mRNA targets for this microRNA in five databases used (Ingenuity Expert Findings, Ingenuity Expert Assist Findings, miRecords, TarBase, TargetScan Human). Three hundred of them are highly predicted and related with metabolic pathways and signaling. The functional classification of miR-708-5p is presented on Table 2. The main signaling pathways modulated by this microRNA were described in cellular growth, proliferation, development and cancer. As seen in Figure 4, dysregulation of mir-708 predicted targets, common to different pathways, have already been associated with CRC establishment and progression. The CREB transcription factor and the CBL oncogene (in complex with MUC1/CIN85) appear to contribute to promotion and progression of CRC [28-30], while the differential expression in the stroma of YWHAZ (a member of the 14-3-3 supports the maintenance of

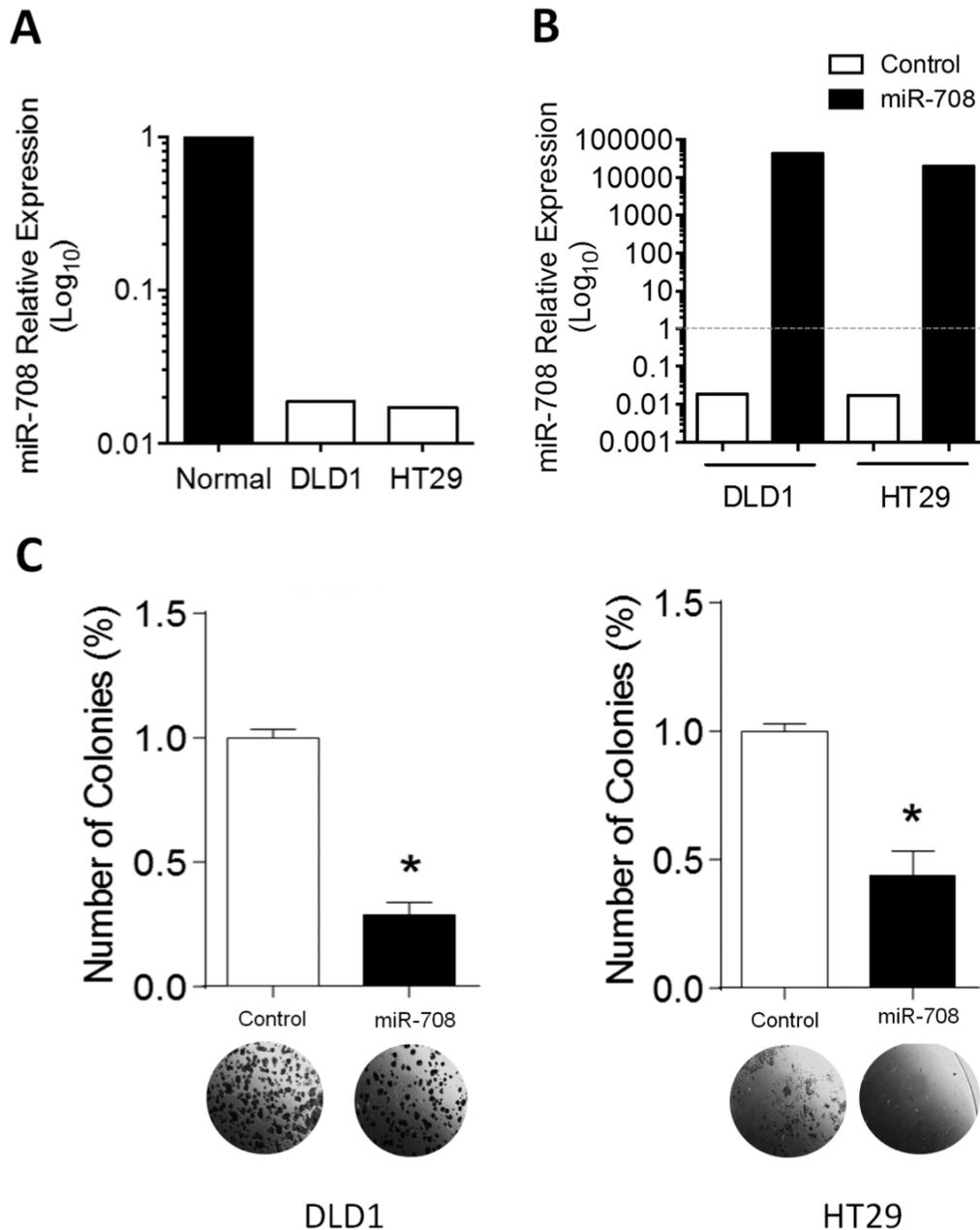


Figure 3: MiR-708-5p forced expression decreases the clonogenic capacity in CRC cells lines. (a) miR-708-5p relative expression in CRC cell lines compared with non-neoplastic tissue; (b) Increased miR-708-5p expression after 48h transfection (42 and 20 thousand fold-change in DLD1 and HT-29 CRC cell lines, respectively) compared to scramble control; (c) Quantitation of the clonogenic assay performed with CRC cell lines expressing miR-708-5p and scramble control with representative images of colonies after 12 days of culture. The data represent median \pm SD (standard deviation) of number cells from four independent experiments. Student's two-tailed t-test was used.

CRC malignant state [31]. Moreover, FOXO1 and GSK3B, both members of the phosphoinositide 3-kinase (PI3K) pathway play pivotal roles in intracellular signal transduction pathways involved in CRC growth, cellular transformation and tumorigenesis [32-33]. Of note, among miR-708 predicted targets is NRAS. Mutations of the RAS family members are hallmarks of CRC [34-35], and their oncogenic activation stimulates a wide range of downstream signaling pathways,

including PI3K/AKT [35-36]. Interestingly, it has been hypothesized that selection for miRNA that coordinately target and regulate multiple members of a cancer-related pathway would be particularly advantageous to tumors [37]. Thus, these *in silico* predictions suggest that miR-708 might act as a tumor suppressor, whose depletion might affect several targets from pathways closely related to CRC maintenance and progression (Figure 5).

Table 2: Description of the Number of miRNAs and their Targets Association with Functional Networks Analyzed Using IPA Software and the IPA Database (Ingenuity Systems, Inc.)

| Ingenuity Canonical Pathways | Number of mRNA-target |
|--|-----------------------|
| Metabolic Pathways | 21 |
| Biosynthesis | 16 |
| Detoxification | 2 |
| Signaling Pathways | 103 |
| Cancer | 40 |
| Apoptosis | 25 |
| Cell Cycle Regulation | 28 |
| Cellular Growth, Proliferation and Development | 43 |
| <i>EGF Signaling</i> | 2 |
| <i>Growth Hormone Signaling</i> | 1 |
| <i>HGF Signaling</i> | 5 |
| <i>IGF-1 Signaling</i> | 5 |
| <i>mTOR Signaling</i> | 2 |
| <i>PI3K/AKT Signaling</i> | 9 |
| <i>STAT3 Pathway</i> | 2 |
| <i>VEGF Signaling</i> | 4 |
| Organismal Growth and Development | 47 |
| <i>Human Embryonic Stem Cell Pluripotency</i> | 8 |
| <i>NF-kB Signaling</i> | 4 |
| <i>Notch Signaling</i> | 0 |
| <i>Sonic Hedgehog Signaling</i> | 1 |
| <i>TGF-beta Signaling</i> | 3 |
| <i>WNT/Ca2+ Pathway</i> | 2 |
| <i>WNT/beta catenin Signaling</i> | 6 |
| Transcriptional Regulation | 8 |

This hypothesis is supported by the decreased clonogenic capacity after restoration of miR-708-5p expression in both CRC cell lines *in vitro*. Although, proliferation and apoptosis were merely affected after induced expression, our results point to a role of this microRNA on colony onset, affecting the reproductive ability of individual cells. Other studies have recently supported the premise of a tumor suppressor role of miR-708-5p. Forced expression of this miRNA in renal cell carcinoma resulted in decreased cell growth and clonogenicity, increased apoptosis, and reduced tumorigenicity in xenograft models [17]. Additionally, reconstitution of miR-708-5p decreased tumorigenicity in prostate cancer [16], and was also able to inhibit cell

proliferation and invasion in glioblastoma [10], providing enhanced evidence for an important role for miR-708-5p down-regulation in carcinogenesis.

Regarding clinical features in CRC patients, our results showed higher expression levels associated with worse prognosis markers, such as presence of grade 3/4 *versus* 1/2; patients with relapse or metastasis *versus* remission patients and in deceased patients group. Additionally, patients with expression values above the median for miR-708-5p were associated with a shorter 2-year event free-survival, suggesting that higher expression levels of this gene may indicate more severe biological effects within CRC tumors. Elevated expression of miR-708-5p has also been associated to high-risk B-cell ALL [12] and poor survival in lung adenocarcinoma [11].

Our results describe low expression in primary tumor tissue compared to normal cells, nevertheless among the CRC patients, lower expression levels may indicate a protective effect related to prognosis. Considering the potential tumor suppressor activity suggested in CRC, we propose that miR-708-5p might differentially contribute to carcinogenesis by regulating dissimilar pathways in less aggressive CRC compared to tumors with higher expression.

On the other hand, miR-708-5p expression analysis was assessed in primary tumor tissues, samples at relapse were unavailable. Changes in miR-708-5p expression levels have been described in ALL samples analyzed at diagnosis and, after treatment in relapse [38]. Differential miR-708-5p expression levels between primary tumor samples and metastases were also recently demonstrated in CRC by microarray expression analysis, showing decreased miR-708-5p expression in metastasis when compared with primary tumors [39]. Even though high miR-708-5p expression in primary tumors is associated with poor prognosis, its expression could be altered with treatment and, in metastatic cells, the miR-708-5p down-regulation could influence the clonogenic capacity. Nonetheless, further studies are needed to corroborate and extend our results.

Taken together, these results suggest that reduced miR-708-5p expression is a common event in human CRC tissues and may be involved in tumor pathophysiology as a tumor suppressor gene. Moreover, miR-708-5p higher expression at primary tumors might represent a biomarker for poor prognosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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