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Original Article

miR-1 and miR-145 act as tumor suppressor microRNAs in gallbladder cancer

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Abstract: The development of miRNA-based therapeutics represents a new strategy in cancer treatment. The objectives of this study were to evaluate the differential expression of microRNAs in gallbladder cancer (GBC) and to assess the functional role of miR-1 and miR-145 in GBC cell behavior. A profile of miRNA expression was determined using Dharmacon™ microarray technology. Differential expression of five microRNAs was validated by TaqMan reverse transcription quantitative-PCR in a separate cohort of 8 tumors and 3 non-cancerous samples. Then, we explored the functional role of miR-1 and miR-145 in tumor cell behavior by ectopic *in vitro* expression in the GBC NOZ cell line. Several miRNAs were found to be aberrantly expressed in GBC; most of these showed a significantly decreased expression compared to non-neoplastic tissues (Q value < 0.05). The differential expression of 7 selected miRNAs was confirmed by real time PCR. Pathway enrichment analysis revealed that the most deregulated miRNAs (miR-1, miR-133, miR-143 and miR-145) collectively targeted a number of genes belonging to signaling pathways such as TGF-β, ErbB3, WNT and VEGF, and those regulating cell motility or adhesion. The ectopic expression of miR-1 and miR-145 in NOZ cells significantly inhibited cell viability and colony formation ($P < 0.01$) and reduced gene expression of VEGF-A and AXL. This study represents the first investigation of the miRNA expression profile in gallbladder cancer, and our findings showed that several miRNAs are deregulated in this neoplasm. *In vitro* functional assays suggest that miR-1 and miR-145 act as tumor suppressor microRNAs in GBC.

Keywords: Gallbladder cancer, microRNAs, miR-1, miR-145, NOZ cells

Introduction

Gallbladder cancer (GBC) is an aggressive neoplasia associated with late diagnosis, unsatisfactory treatment and poor prognosis [1, 2]. An early diagnosis is rarely achieved because the signs and symptoms of GBC are not specific and often appear late in the clinical course of the disease. For this reason, the diagnosis is generally made when the cancer is already at an advanced stage [3]. Coadjuvant therapy with chemo- and/or radiotherapy has not had the hoped-for impact on the survival of patients with advanced GBC [4]. There is therefore an urgent need to identify molecular targets for therapy.

MicroRNAs (miRNAs) are small non-coding RNAs approximately 18-25 nucleotides in length that negatively regulate gene expression [5]. The traditional dogma has been that these small molecules can cause either mRNA degradation or inhibit translation. When binding to its target mRNA with complete complementarity, the miRNA can lead to degradation of the target. miRNAs can also bind to their targets with incomplete complementarity, often in the 3'-UTR regions, and this leads to the translational suppression of their target genes [6-8].

miRNAs are expressed in a tissue-specific manner and play important roles in the regulation of a large number of essential biological functions

that are critical to normal development, including cell proliferation, differentiation, apoptosis, metabolism and immune response [9, 10]. Therefore, the deregulation of their expression may have negative effect on normal cell growth, contributing to the development of diseases such as diabetes, immuno- or neurodegenerative disorders and cancer [11-13]. Many studies have demonstrated that the loss and gain of function of specific miRNAs may be key events in oncogenesis and they can act as either oncogenes (if their genes are amplified or hyper expressed) or tumor-suppressors (in case of deletions or repressions of miRNAs), by acting alone or clustered [14, 15]. Many miRNAs are currently under investigation as diagnostic and prognostic biomarkers, therapeutic targets and as markers of cancer subtypes [16-18].

An aberrant expression of microRNAs has been reported for multiple cancers, such as colon cancer [19], pancreatic cancer [20], gastric cancer [21], breast cancer [22], melanoma [23, 24], papillary thyroid carcinoma [25] and cholangiocarcinoma [26, 27]. To our knowledge, only two studies have analyzed the implications of miRNAs in gallbladder cancer [28, 29]. In this study, we identified differentially expressed microRNAs in GBC and validated the *in vitro* functional effect of miR-1 and miR-145, two miRNAs downregulated in GBC. Our findings provide a better understanding of gallbladder cancer biology, and may lead to the development of novel therapeutic applications that complement and enhance the current management of this cancer.

Materials and methods

Gallbladder tissues

A total of 21 gallbladder tissues were included in this study. The microarray experiment was done with 4 non-neoplastic (normal) and 6 tumor tissues, whereas the subsequent quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) validations included 3 normal tissues and 8 tumors (different cohort). The normal samples were obtained from patients undergoing surgery for reasons unrelated to cancer. All gallbladder samples were frozen tissues collected at the time of diagnosis through an approved tissue collection protocol at the Universidad de La Frontera, Temuco, Chile.

Gallbladder cancer cell lines

MicroRNA expression was evaluated in nine human GBC cell lines (GB-d1, G-415, SNU-308, OCUG-1, NOZ, TGBC14, TGBC24, TGBC-1TKB and TGBC-2TKB) and one of them (NOZ) was selected for performing functional assays. GB-d1, G-415 and SNU-308 were provided by Dr. Anirban Maitra (Department of Pathology, Johns Hopkins University School of Medicine, USA); OCUG-1 and NOZ from Japan Health Science Research Resources Bank (HSRRB) and TGBC14, TGBC24, TGBC-1TKB and TGBC-2TKB from RIKEN Bio Resource Center.

Cell culture conditions

G-415, GB-d1 and SNU-308 were grown in RPMI 1640 medium (Thermo Scientific Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 10 units/ml penicillin and 10 mg/ml streptomycin (1% P/S) (Thermo Scientific Hyclone). OCUG-1, TGBC-24 and TGBC-14 was cultured in DMEM high glucose and NOZ in Williams' E medium (Invitrogen, Life Technologies Corporation, Grand Island, NY, USA) with 10% FBS and 1% P/S. TGBC-1TKB and TGBC-2TKB were grown in DMEM high glucose supplemented with 5% FBS and 1% P/S. All nine cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and were subcultured during the logarithmic phase.

RNA purification

Total RNA was extracted from gallbladder tissues and GBC cell lines using the mirVana miRNA extraction kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

MicroRNA expression profiling

The miRNA microarray experiments were performed by Thermo Scientific Dharmacon miRNA profiling service. This platform utilized two-color high-density 8-plex slides comprised of probes to capture all human, mouse and rat mature microRNAs annotated at miRBase Sequence Database (Release 10.1 - December 2007). The data processing service included calculation of relative intensity, error and *P*-values for each probe. For microarray data

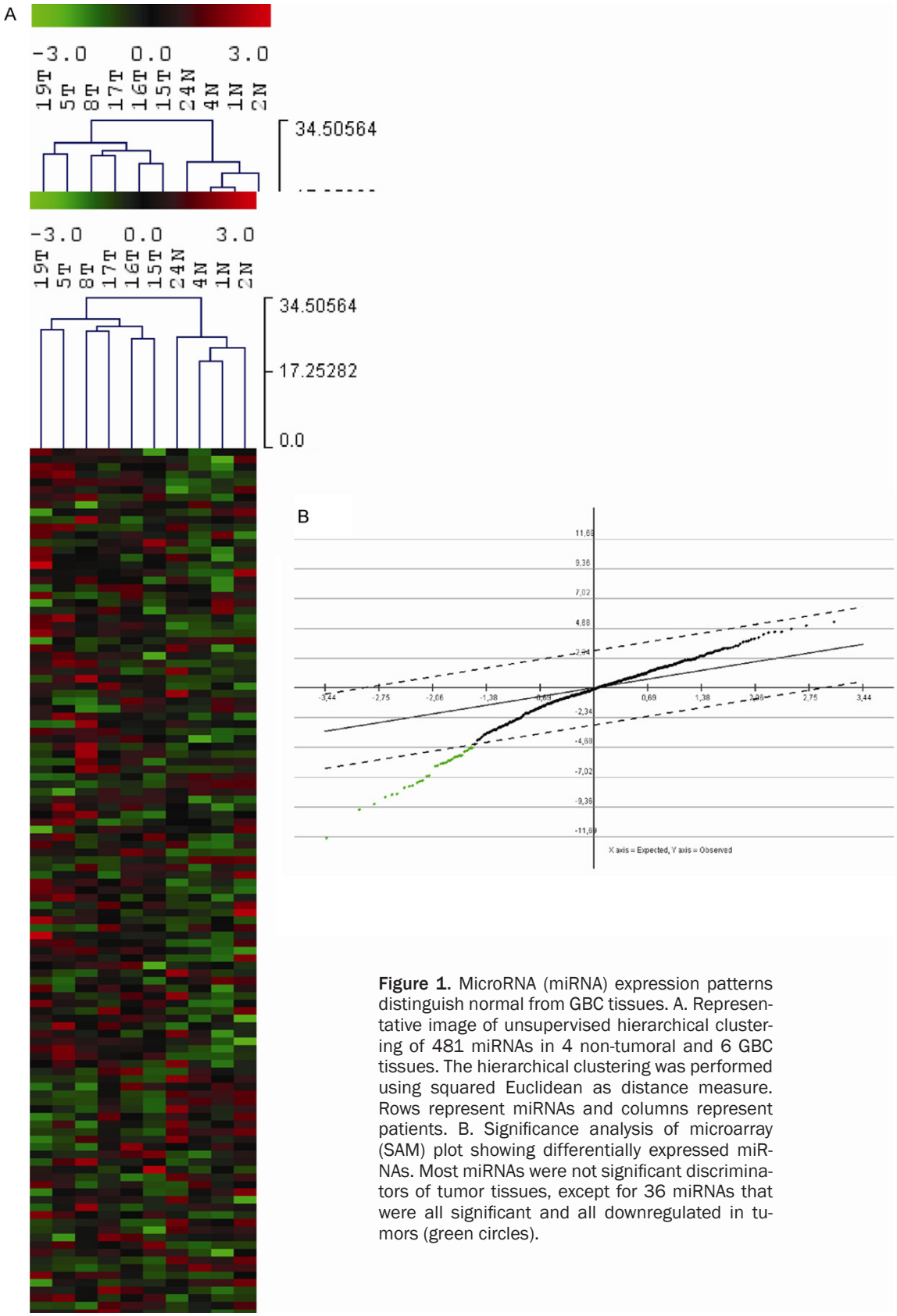

Figure 1. MicroRNA (miRNA) expression patterns distinguish normal from GBC tissues. A. Representative image of unsupervised hierarchical clustering of 481 miRNAs in 4 non-tumoral and 6 GBC tissues. The hierarchical clustering was performed using squared Euclidean as distance measure. Rows represent miRNAs and columns represent patients. B. Significance analysis of microarray (SAM) plot showing differentially expressed miRNAs. Most miRNAs were not significant discriminators of tumor tissues, except for 36 miRNAs that were all significant and all downregulated in tumors (green circles).

Table 1. MicroRNAs downregulated in GBC tissues

miRCode	Log(2) Fold-Change[a]	Chromosomal location
hsa-miR-133a; hsa-miR-133b	-3.58	18q11.2 (a-1); 20q13.33 (a-2); 6p12.2 (b)
hsa-miR-143-3p	-3.18	5q32
hsa-miR-145-5p	-2.81	5q32
hsa-miR-99a-5p	-2.52	21q21.1
hsa-miR-125b-5p	-2.46	11q24.1 (b-1); 21q21.1 (b-2)
hsa-miR-1	-2.03	20q13.33 (-1); 18q11.2 (-2)
hsa-miR-29c-3p	-1.98	1q32.2
hsa-miR-195-5p	-1.86	17p13.1
hsa-miR-139-5p	-1.74	11q13.4
hsa-miR-29c-5p	-1.61	1q32.2
hsa-miR-100-5p	-1.58	11q24.1
hsa-miR-143-5p	-1.53	5q32
hsa-miR-148a-3p	-1.42	7p15.2
hsa-miR-145-3p	-1.32	5q32
hsa-miR-376c	-1.29	14q32.31
hsa-miR-187-3p	-1.27	18q12.2
hsa-miR-365a-3p	-1.24	16p13.12 (a); 17q11.2 (b)
hsa-miR-29b-3p	-1.24	7q32.3 (b-1); 1q32.2 (b-2)
hsa-miR-497-5p	-1.20	17p13.1
hsa-miR-654-3p	-1.20	14q32.31
hsa-miR-411-5p	-1.12	14q32.31
hsa-miR-125a-5p	-1.10	19q13.41
hsa-miR-26a-5p	-1.09	3p22.2 (a-1); 12q14.1 (a-2)
hsa-miR-101-3p	-1.08	1p31.3 (-1); 9p24.1 (-2)
hsa-miR-495	-1.05	14q32.31
hsa-miR-381-3p	-1.00	14q32.31
hsa-miR-154-5p	-0.96	14q32.31
hsa-miR-99a-3p	-0.88	21q21.1
hsa-miR-328	-0.87	16q22.1
hsa-miR-299-5p	-0.85	14q32.31
hsa-miR-30e-3p	-0.83	1p34.2
hsa-miR-29b-2-5p	-0.80	1q32.2
hsa-miR-379-5p	-0.76	14q32.31
hsa-miR-140-5p	-0.74	16q22.1
hsa-miR-24-1-5p	-0.59	9q22.32
hsa-miR-101-5p	-0.49	1p31.3

[a]Fold changes were calculated by comparison of the mean expression value in the tumor group to the control group (normal gallbladder tissues).

analysis, unsupervised hierarchical clustering was carried out to generate the tree structures of samples and miRNAs, using the Multi Experiment Viewer (MEV) packages v. 4.9 [30]. An average linkage clustering analysis of the data was based on Euclidean distance. Differentially expressed miRNAs between disease and normal tissues were identified using the Significance Analysis of Microarrays (SAM) [31]. The cutoff for significance was determined by a tuning parameter delta, chosen based on the false discovery rate (FDR) and represented by the Q value.

In silico pathway analyses

The potential function of validated and differentially expressed miRNAs in biological processes

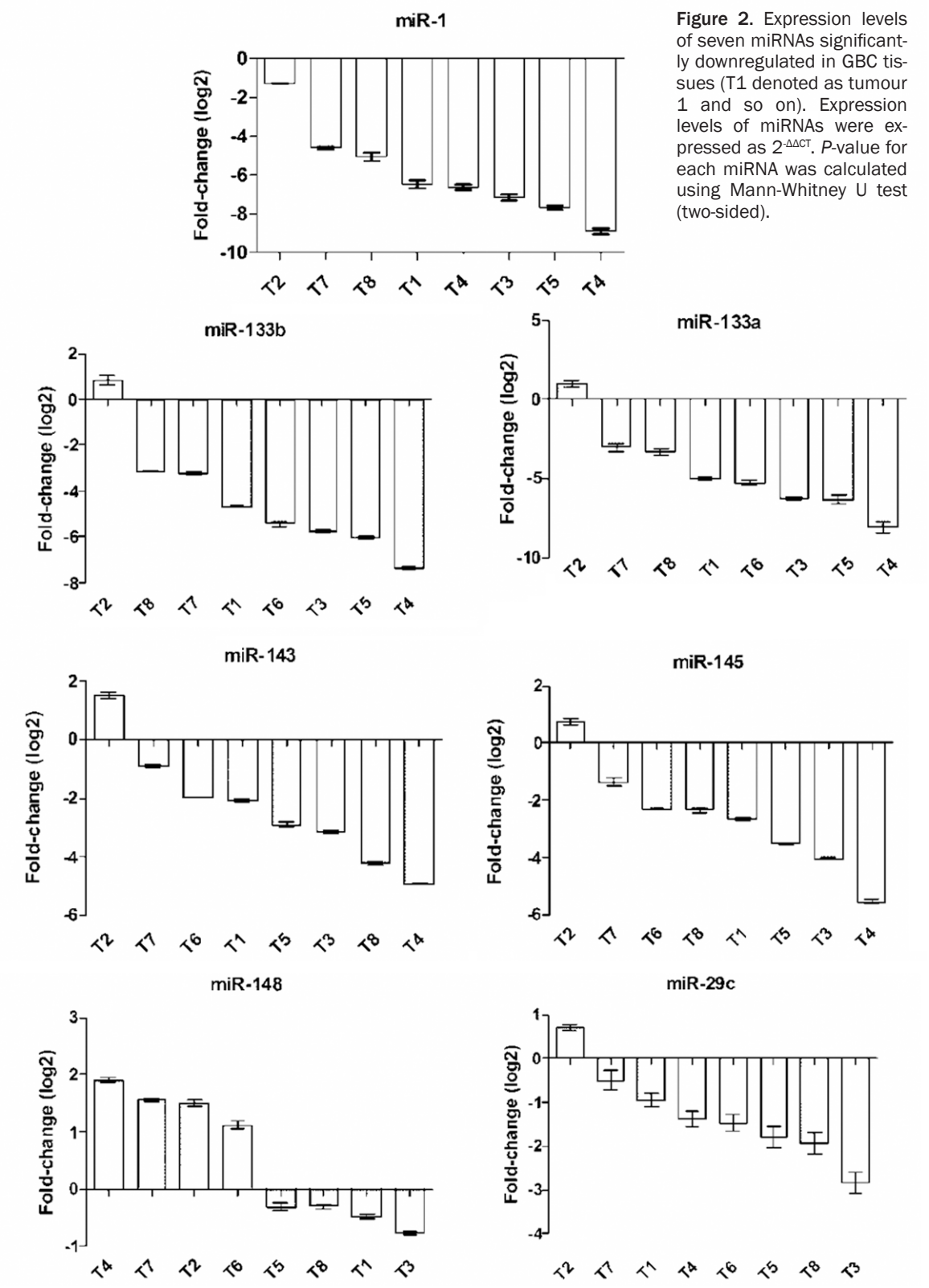

Figure 2. Expression levels of seven miRNAs significantly downregulated in GBC tissues (T1 denoted as tumour 1 and so on). Expression levels of miRNAs were expressed as $2^{-\Delta\Delta CT}$. *P*-value for each miRNA was calculated using Mann-Whitney U test (two-sided).

was explored using on-line bioinformatics resources. TargetScan algorithm (release 6.2) was used to search for predicted microRNA targets in human microRNA database (http://

Table 2. Comparison of microarray and qPCR results for microRNA expression

	Log(2) Fold-Change[a]	
miRCode	Microarray	qPCR
hsa-miR-133a	-3.58	-4.55
hsa-miR-133b	-3.58	-4.36
hsa-miR-143-3p	-3.18	-2.33
hsa-miR-145-5p	-2.81	-2.62
hsa-miR-1	-2.03	-5.98
hsa-miR-29c-3p	-1.98	-1.27
hsa-miR-148a-3p	-1.42	0.53

[a]Fold changes were calculated by comparison of the mean expression value in the tumor group to the control group (normal gallbladder tissues). Abbreviation: qPCR, quantitative real-time polymerase chain reaction.

www.targetscan.org). Low confidence targets were eliminated by filtering out targets with total context score greater than -0.2. The predicted targets were incorporated into an integrated gene ontology database molecular annotation system (Molecule Annotation System (MAS) v3.0, http://www.capitalbio.com) to investigate their involvement in different signaling pathways.

Quantitation of microRNAs by real time RT-PCR

The expression of selected significantly deregulated miRNAs was validated in a different set of samples (8 gallbladder tumors and 3 normal tissues) and in GBC cell lines. The relative expression level of each selected miRNA was quantified using the TaqMan MicroRNA Assay (Life Technologies, Foster city, CA), following the protocol provided by the manufacturer. Expression data for miRNA were acquired using Stratagene Mx3000P QPCR System (Agilent Technologies Inc., Santa Clara, CA, USA). Small RNA RNU6B was used as an internal control for input normalization. The cycle number at which the real-time PCR reaction reached a determined threshold (Ct) was recorded for both the miRNAs and RNU6B, and the relative miRNA expression was expressed as $2^{-\Delta\Delta CT}$. Mann-Whitney U test was used for comparison between the two groups (normal and tumor) at a significance level of 0.05 (two-sided).

Cell transfection and controls

Transfection of NOZ cells was performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Foster city, CA) according to manufacturer's instructions. Briefly, NOZ cells were seeded at 7×10^4 in 6-well culture plates. After 24 h (70% confluence), cells were transfected with 50 nM of pre-miR-1, (5'UGGAAUGUAAAGAAGUAUGUAU'3) and 50 nM of pre-miR-145-5p (5'GUCCAGUUUUCCCAGGAAUCCCU'3) (mirVana™ mimics & controls; Ambion, Austin, TX). Non-specific pre-miR (*mir*Vana™ miRNA mimic) and siRNA (BLOCK-iT™ Alexa Fluor® Red Fluorescent) were used as negative (scrambled) and positive control, respectively. The success of transfection was confirmed by quantitative real-time PCR and visualization of positive control by confocal microscopy. These controls were used under the same experimental conditions that mimic microRNAs and allowed to assess cell transfection efficiency during each assay.

Cell viability assay

Cell viability was evaluated using the tetrazolium-based MTS assay (CellTiter 96 AQueous One Solution Reagent; Promega, Madison, USA). Briefly, transfected NOZ cells were seeded onto 96-well plates at 2500 cells per well. After 4, 24, 48, 72 and 96 hours, 20 µl of MTS reagent was added to each well and the cells were incubated for an additional hour. Mitochondrial dehydrogenases of viable cells reduced the yellow MTS to the blue formazan dye, the absorbance of which correlates to the amount of viable cells. Absorbance was measured at 490 nm using an automated microplate reader (Autobio Labtec Instruments Co. Ltd., Zhengzhou City, China). Each condition was tested in triplicate from which mean values and SEM were calculated.

Colony formation assay

For colony formation assay, cells were transfected with miRNA mimics or controls for 24 hours, and then seeded in 6-well plate in triplicate. After 10 days of incubation, plates were gently washed with PBS and stained with 0.5% of crystal violet in 25% methanol/PBS for one hour at room temperature. Colonies with over 50 cells were manually counted.

Cell cycle and apoptosis analysis

For cell cycle and apoptosis analysis by flow cytometry, NOZ cells were plated into 6-well plates 24-hours prior to transfection with mirVana™ mimics and controls. After 72 hours, apoptosis was quantified by double staining

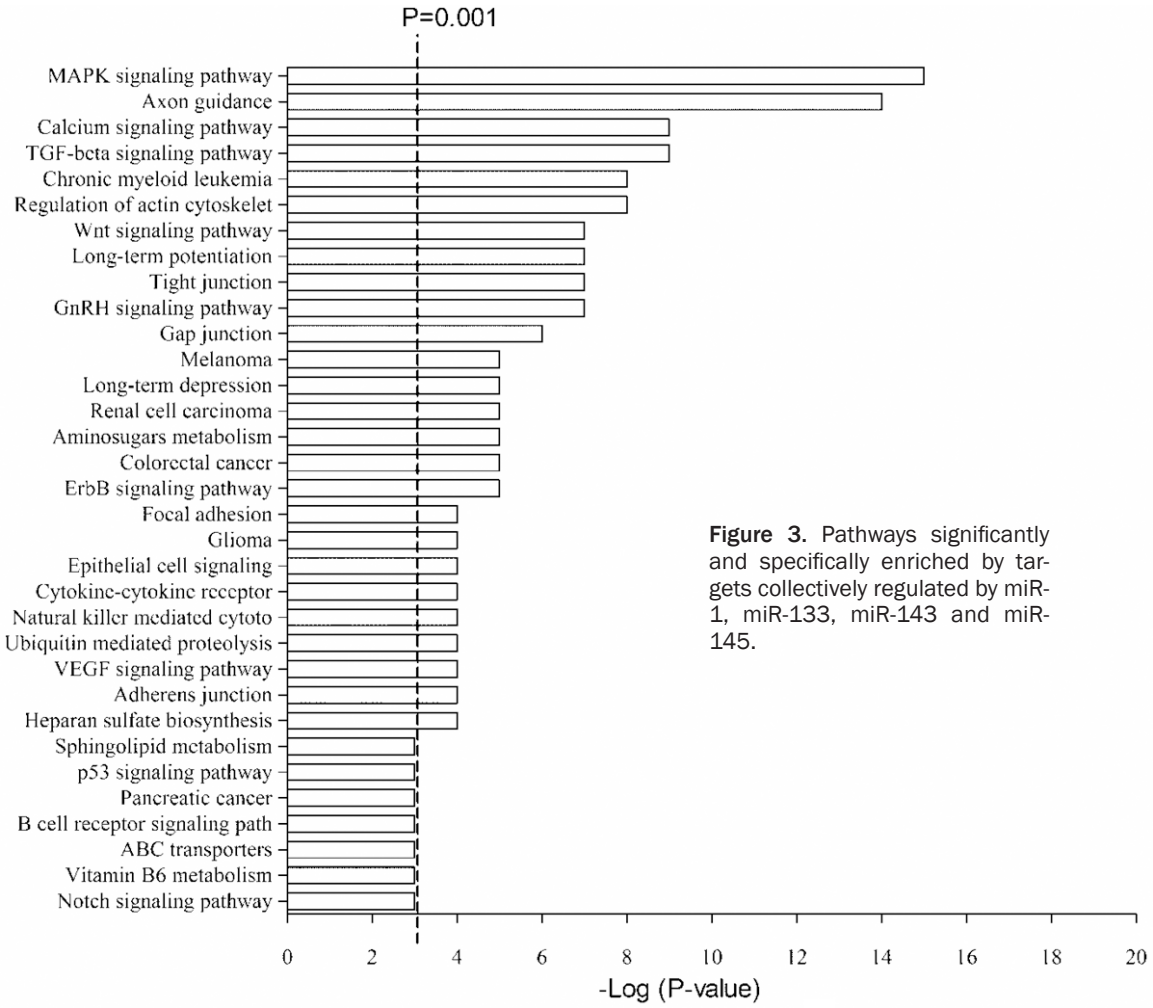

Figure 3. Pathways significantly and specifically enriched by targets collectively regulated by miR-1, miR-133, miR-143 and miR-145.

with Annexin V/Propidium Iodide (Apoptosis Assay Annexin V and Propidium Iodide kit, Biotium). Briefly, cells were harvested and suspended at 5-10 × 10^6 cells/mL in 1 × Binding Buffer. The cell suspension (100 µl) was incubated with 5 µl of CF 488A-Annexin V and 2 µL of propidium iodide at room temperature for 30 minutes in the dark. The stained cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). For the flow cytometry analysis, 72 hours post-transfection, cells were trypsinized and fixed in 70% ice-cold ethanol for 2 hours. After centrifugation, cells were stained with 10 µg/ml propidium iodide and 100 µg/ml RNase A, and then analyzed by flow cytometry.

miRNA target analysis

In silico prediction of miRNA binding sites within the 3'UTR of highly expressed genes in GBC (VEGF-A, AXL and ErbB3) was performed using miRecords (http://miRecords.umn.edu/miRecords) (data not shown). The mRNA expression of VEGF-A, AXL and ErbB3 was analyzed by quantitative RT-PCR after 24, 48 and 72 hours post-transfection. Briefly, RNA was reverse transcribed with random primers at 42°C for 50 min using M-MLV reverse transcriptase 200 U/µl (Promega Corp., Madison, WI, USA). The resulting cDNA was subsequently amplified by PCR using the Brilliant II Ultra-Fast SYBR® Green qPCR Master Mix according to the manufacturer's recommendation on Stratagene Mx-3000p Real-Time PCR System (Agilent Technologies Inc., Santa Clara, CA, USA). Relative fold levels were determined using the $2^{-\Delta\Delta CT}$ method, with β-Actin used as housekeeping control. Primer pairs used were (primer sequences 5'-3'), for VEGF-A, forward TGGACTTGAGTTGGGAGGGGAAT and reverse CGGTGTTCCCAAAACTGGGTCATA, for TWFL-1, forward TGGGGATGAGTTGACTGCAGACTT and reverse

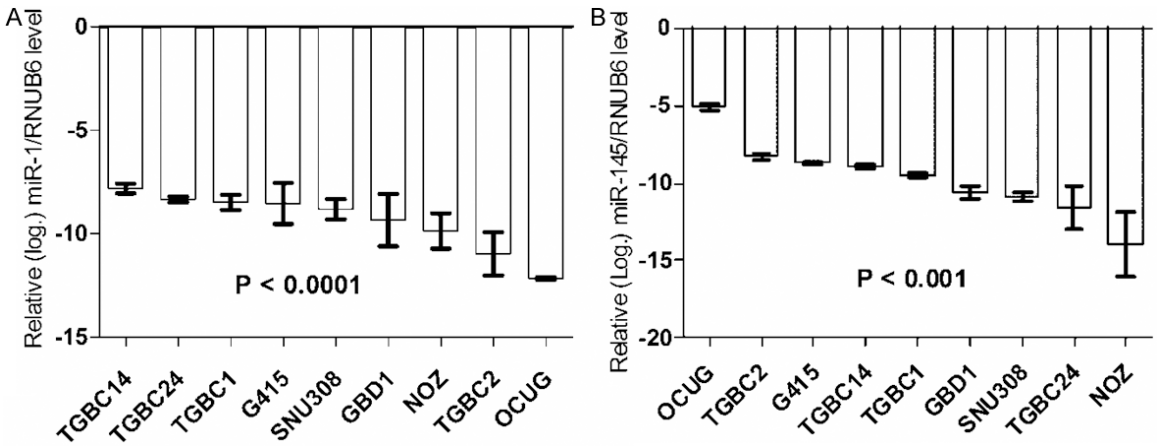

Figure 4. Relative expression of miR-1 (A) and miR-145 (B) in gallbladder cancer cell lines. Values were normalized with RNUB6 and further to the expression level of normal gallbladder epithelium. The mean level of relative expression in the nine cell lines was compared to normal tissues using an unpaired two-tailed Student's *t*-test. The error bars indicate the standard error.

TTCCGCTGGGCCCCTAATTAGT, for AXL, forward CCAGTCAAGTGGATTGCCATTGAG and reverse GATTTCCCTGGCGCAGATAGTCAT, for ErbB3, forward CCATGTCCATTATGCCCGCCTAAA and reverse GCTGCCATTAAATGCTCCCTGAGT and for β-actin, forward GACAGGATGCAGAAGGAGATTACT and reverse TGATCCACATCTGCTGGAAGGT. Primers were tested to determine their optimal concentrations for PCR analysis and the resulting products were run on 2% agarose gel to confirm the appropriate size. Efficiency of real-time PCR reaction was calculated from standard curves.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). All experiments were repeated at least three times and performed in triplicate. Means were compared using the two-tailed Student's *t*-test, two-tailed Mann Whitney *U* test, or two-ways ANOVA. A *P*-value of 0.05 was considered statistically significant.

Results

miRNA expression profile in gallbladder cancer

A total of 481 miRNAs were detectable after microarray hybridization. Unsupervised hierarchical clustering analysis using expression levels of all 481 detected miRNAs in normal and GBC tissues generated a tree with normal and GBC samples clearly separated into two groups (**Figure 1A**). Using the significance analysis of microarrays (SAM) algorithm, we identified a set of 36 miRNAs consistently downregulated in GBC (none up-regulated) at Q = 0 and fold-change > 1.0, as compared to normal gallbladder mucosa (**Figure 1B**). The mature sequences representing miR-133a/133b and miR-143 were the most highly decreased in GBC tissues (-3.6-fold and -3.2-fold respectively). A complete list of these differentially downregulated miRNAs sorted by fold-change ratio is shown in **Table 1**.

Validation of microarray data by real-time RT-PCR

To validate the microarray results, a set of 7 miRNAs (hsa-miR-133a; hsa-miR-133b; hsa-miR-143; hsa-miR-145; hsa-miR-1, has-miR-148 and hsa-miR-29c) was chosen for analysis by qRT–PCR in an independent set of 3 normal and 8 GBC tissues. Fold changes were normalized to the level of RNU6B expression, and relative to the mean expression of the normal tissues. The qRT-PCR results demonstrated high concordance with the miRNA microarray data, except for hsa-miR-148a (**Figure 2**; **Table 2**). The relative miRNA expression varied between tumors; only one tumor (T2) showed a different behavior in relation to miRNA expression, which was elevated relative to the normal control. This case did not have any pathologically or clinically distinctive features, among those recorded in the database, to explain this difference. The Dharmacon miRNA array used in this

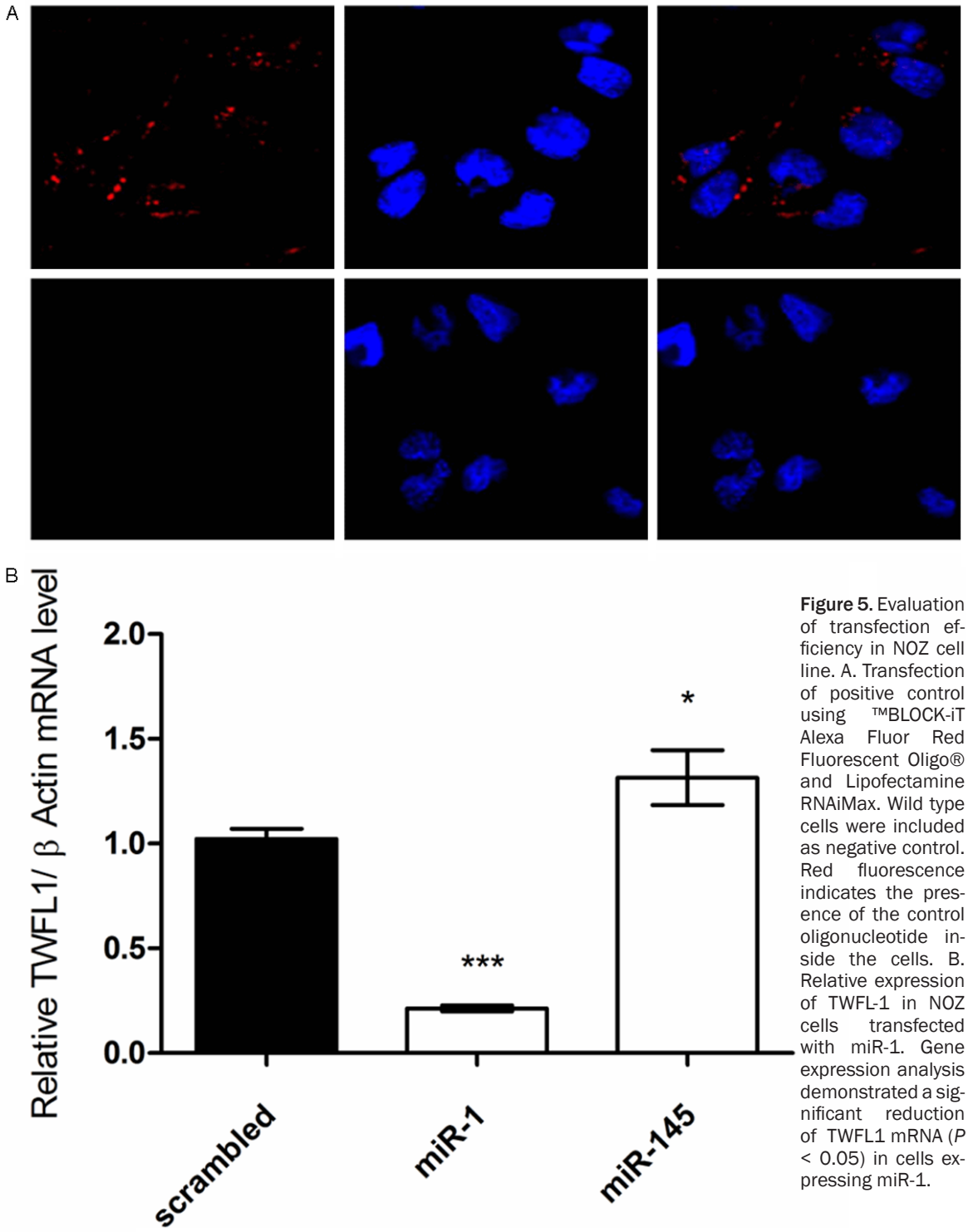

Figure 5. Evaluation of transfection efficiency in NOZ cell line. A. Transfection of positive control using ™BLOCK-iT Alexa Fluor Red Fluorescent Oligo® and Lipofectamine RNAiMax. Wild type cells were included as negative control. Red fluorescence indicates the presence of the control oligonucleotide inside the cells. B. Relative expression of TWFL-1 in NOZ cells transfected with miR-1. Gene expression analysis demonstrated a significant reduction of TWFL1 mRNA ($P < 0.05$) in cells expressing miR-1.

study does not distinguish between hsa-miR-133a and hsa-miR-133b; however, the Taqman miRNA assay uses specific probes to detect each of these miRNAs. The results showed that both miRNAs are downregulated in GBC, compared to non-neoplastic tissues (**Figure 2**).

Gene ontology and signaling pathways analysis

The potential function of the downregulated miRNAs hsa-miR-1, hsa-miR-133a/b, hsa-miR-143 and hsa-miR-145 on cellular processes

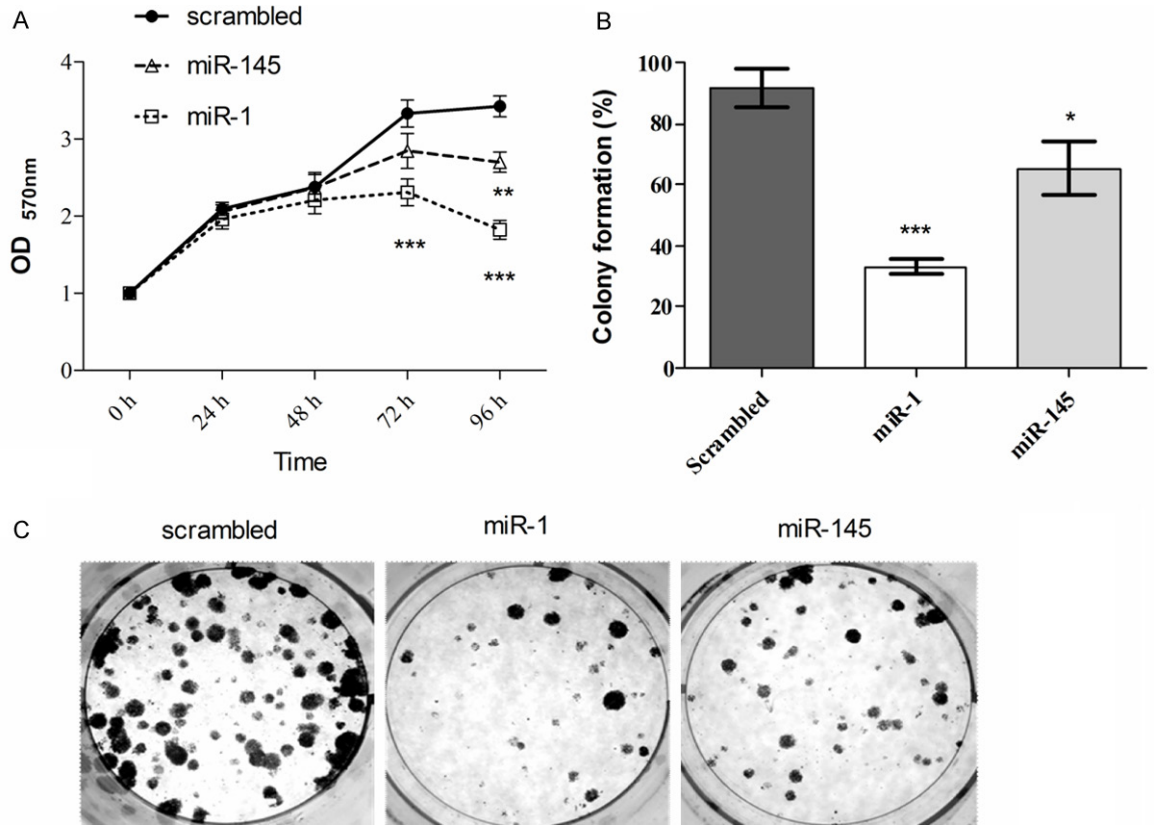

Figure 6. Ectopic expression of miR-1 and miR-145 in NOZ cells decreases cell viability and colony formation *in vitro*. A. NOZ cells expressing miR-1 and miR-145 showed a decreased viability compared to cells expressing the scrambled control. The means were compared using an unpaired two-tailed Student's t-test.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The error bars indicate the standard error. B. The percentage of colonies significantly decreased in cells expressing miR-1 and miR-145. Values were compared to scrambled control using a 2-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The error bars indicate the standard error. C. Representative image of colony formation assay of NOZ cells expressing microRNA mimics.

was explored through *in silico* pathway analysis using the MAS (Molecule Annotation System) platform. These miRNAs were chosen because they showed a median fold-change > 2.0 in gallbladder tumors (**Table 2**). Targets were predicted using the TargetScan algorithm, considering only those targets with a context score greater than -0.2. The predicted targets were used to identify biological processes that could be collectively regulated by these four miRNAs. The KEGG pathways were sorted by the enrichment *P*-value calculated by MAS. Thirty-three KEGG annotations were found to be statistically enriched ($P < 0.001$). Many of these potentially altered signaling pathways are involved in tumor pathogenesis, such as MAPK, TGF-β, Wnt, ErbB, VEGF and Notch, and those regulating cell motility or adhesion (**Figure 3**). Gene association using the gene ontology database (GO) showed that genes potentially regulated by these microRNAs participate in different biological processes associated with human cancer, including transcription regulation, signal transduction, positive regulation of cell proliferation, cell adhesion and angiogenesis, among others (data not shown).

miR-1 and miR-145 are downregulated in GBC cell lines

Among the validated miRNAs, it is recognized that downregulation of miR-1 and miR-145 is associated with aggressive behavior of cancer cells in digestive neoplasias [32-39]. Therefore, we evaluated the possible implications of these miRNAs in gallbladder cancer. First, we examined the expression of miR-1 and miR-145 in 9 human gallbladder cancer cell lines and then we performed *in vitro* assays to determine the potential role of these miRNAs. As shown in **Figure 4**, miR-1 and miR-145 expression was significantly decreased in GBC cell lines ($P <$

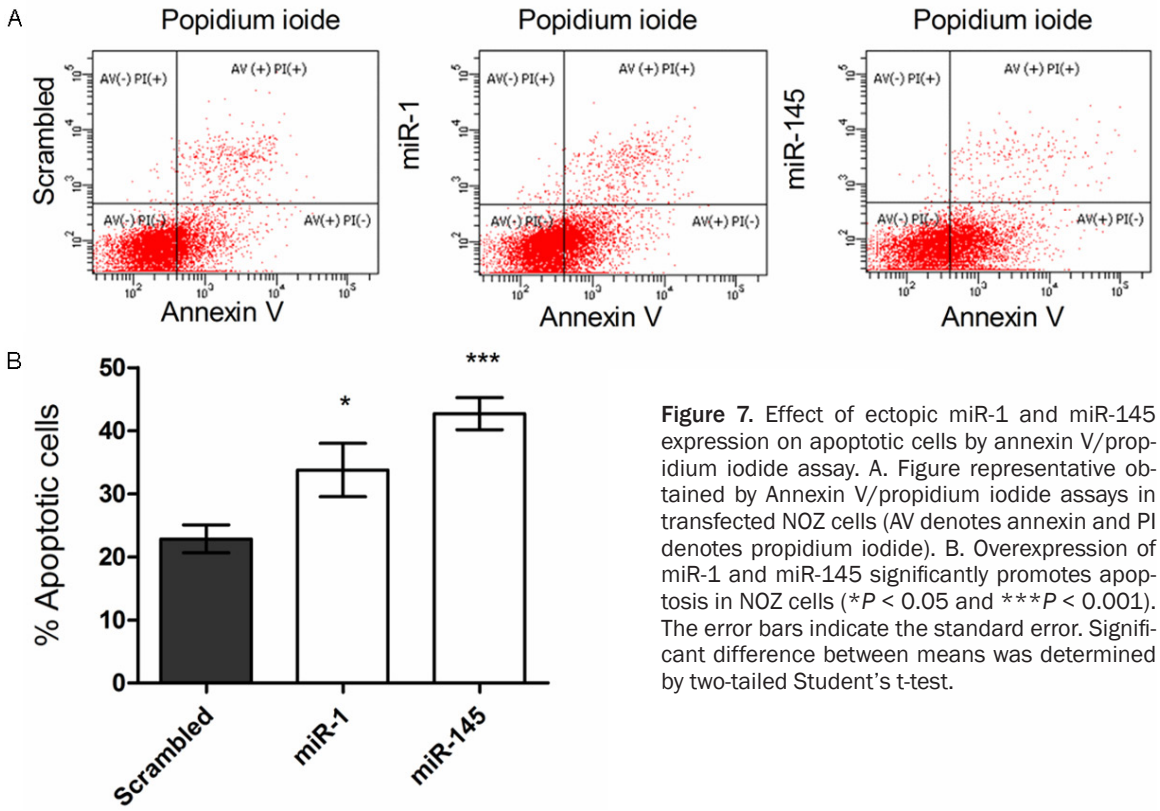

Figure 7. Effect of ectopic miR-1 and miR-145 expression on apoptotic cells by annexin V/propidium iodide assay. A. Figure representative obtained by Annexin V/propidium iodide assays in transfected NOZ cells (AV denotes annexin and PI denotes propidium iodide). B. Overexpression of miR-1 and miR-145 significantly promotes apoptosis in NOZ cells (*$P < 0.05$ and ***$P < 0.001$). The error bars indicate the standard error. Significant difference between means was determined by two-tailed Student's t-test.

Table 3. Cell cycle analysis of NOZ cell line expressing miR-1 and miR-145

	G0/G1	S	G2/M
Scrambled	35.75 ± 1.31	7.00 ± 0.82	57.25 ± 2.10
miR-1	34.75 ± 1.49	9.00 ± 1.23	56.00 ± 0.58
miR-145	36.50 ± 0.96	6.25 ± 0.25	57.25 ± 0.63

Results are expressed as the percentage of total cells. Data represent mean ± SD values of triplicate experiments.

0.001) compared to normal gallbladder tissues. Using NOZ gallbladder cancer cells as an *in vitro* model, we evaluated the functional role of miR-1 and miR-145 by transfecting cells with pre-miR miRNA precursor molecules. Transfections were controlled by measuring miRNA expression levels by qRT-PCR and visualizing the positive control (BLOCK-iT™ Alexa Fluor® Red Fluorescent). Cells were transfected with 15 pmol of positive control (24-well format) and after 72 hours were harvested, fixed with 4% paraformaldehyde and stained with DAPI (0.1 μg/ml) for visualization of the nucleus. The fluorescence was observed under the Olympus spectral confocal microscope. High transfection efficiency was observed (**Figure 5A**). In addition, the activity of miR-1 in transfected cells was monitored through the expression of twinfilin actin-binding protein 1 (TWFL-1 or PTK9), a recognized target of miR-1, relative to scrambled (**Figure 5B**). Relative expression of both microRNAs increased over 15 times compared to scrambled cells and was stable during the evaluated period (24, 48, 72 and 96 hours post-transfection; data not shown).

mir-1 and miR-145 inhibit NOZ cell growth

miR-1 and miR-145 dramatically reduced NOZ cell viability compared to scrambled control (**Figure 6A**). The inhibitory effect on cell growth was further confirmed by colony formation assay. The number of colonies was significantly reduced in cells transfected with miR-145 and miR-1 mimics (**Figure 6B**), compared to cells transfected with scrambled. The percentage of inhibition was 64% for miR-1 ($P < 0.001$) and 29% for miR-145 ($P < 0.05$).

miR-1 and miR-145 induce apoptosis without affecting the cell cycle

Inhibition of cell growth in cancer cells is usually associated with concomitant cell cycle arrest and activation of cell death pathways. We therefore examined the contribution of apoptosis and cell cycle arrest to growth inhibition mediated by miR-1 and miR-145 expres-

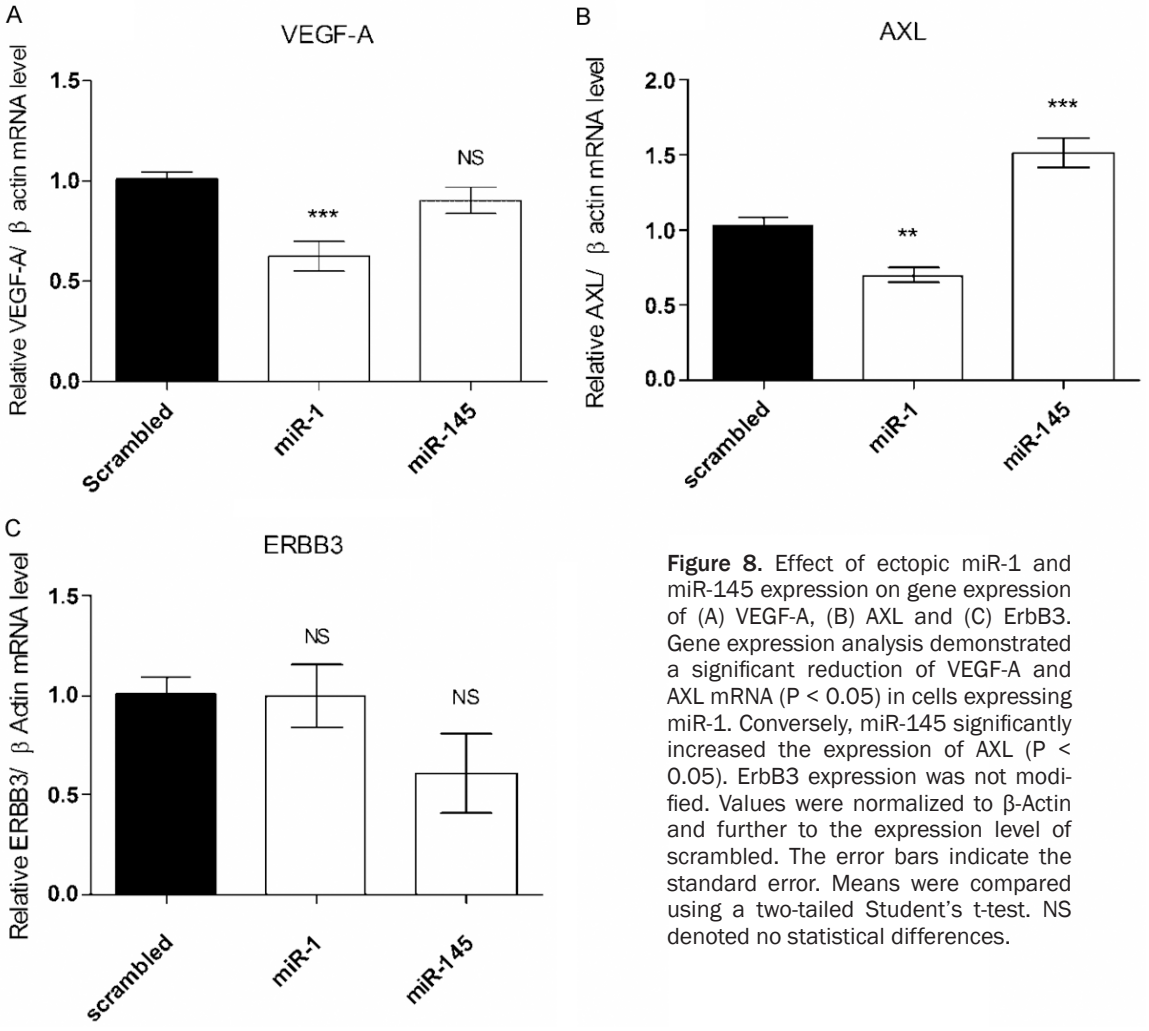

Figure 8. Effect of ectopic miR-1 and miR-145 expression on gene expression of (A) VEGF-A, (B) AXL and (C) ErbB3. Gene expression analysis demonstrated a significant reduction of VEGF-A and AXL mRNA ($P < 0.05$) in cells expressing miR-1. Conversely, miR-145 significantly increased the expression of AXL ($P < 0.05$). ErbB3 expression was not modified. Values were normalized to β-Actin and further to the expression level of scrambled. The error bars indicate the standard error. Means were compared using a two-tailed Student's t-test. NS denoted no statistical differences.

sion. The overexpression of miR-1 and miR-145 in NOZ cells promotes apoptosis compared with scrambled (**Figure 7A**). The positive average percentage for Annexin V was of 34% for miR-1, 43% for miR-145 and 23% in cells control (**Figure 7B**). The propidium iodide staining showed very low positivity in all groups (alone or combined with Annexin V) and no statistically significant difference between groups. In order to monitor the influence of miR-1 and miR-145 on cell cycle, we quantified the percentage of cells in different cell cycle phases by flow cytometry. At 72 hours after transfections, over 50% of cells were in G2/M phase and no significant differences between groups were observed (**Table 3**).

Expression of miR-1 inversely correlated with gene expression of VEGF-A and AXL

Based on the bioinformatics prediction we examined the effect of miR-1 and miR-145 on mRNA expression of three potential targets (VEGF-A, AXL and ErbB3). This assay was conducted in NOZ cells previously transfected with scrambled and microRNAs mimics. As shown in **Figure 8**, VEGF-A and AXL mRNA expression dramatically decreased in miR-1 transfected cells compared with miRNA control-transfected cells ($P < 0.0001$; $P < 0.001$ respectively). The expression of VEGF-A mRNA was not altered by miR-145 and interestingly the AXL was significantly increased ($P < 0.0001$). Gene expression of ErbB3 was not affected by any of the two miRNAs studied (**Figure 8C**).

Discussion

miRNAs regulate many key biological processes by post-transcriptional control of multiple genes that determine the function of the cells under homeostatic and disease conditions [40]. Regulation processes such as inflamma-

tion, cell cycle regulation, stress response, cell differentiation, apoptosis and invasion which may directly affect the development and tumor progression [41]. Thus, they have been afforded great potential as biomarkers and therapeutic targets, which have been shown to restore normal expression level and can reduce and even eliminate tumors in animal models [42].

In this study we conducted a comprehensive miRNA expression profiling by analyzing the expression levels of human miRNAs in 6 GBC and 4 normal gallbladder tissues. The miRNA expression profiling separated the samples into two major clusters distinguishing between normal and tumors. We found that several miRNAs are significantly deregulated in gallbladder, most of them showing a decreased expression compared to non-neoplastic tissues. Among them, miR-1 and miR-145 had a significantly lower expression in gallbladder cancer than normal tissue (validated results by qRT-PCR). Therefore, we assessed the expression of miR-1 and miR-145 in cell lines of gallbladder cancer by real time PCR. We also studied the effect of ectopic expression of miR-1 and miR-145 on cell growth *in vitro* and the gene expression level of potential targets (VEGF-A, ErbB3 and AXL). Our results confirmed a lower expression of these miRNAs in GBC cell lines than in normal gallbladder tissues; this result is consistent with multiple studies that have reported a decrease in the expression of miR-145 and miR-1 in human tumors. miR-145 is downregulated in colon cancer [43], in B cell neoplasms [44], on ACTH-secreting pituitary adenomas [45], breast cancer [46] and miR-1 in lung cancer [47], rhabdomyosarcoma [48], squamous cell carcinoma of head and neck [49] and in hepatocarcinoma [33]. The only exception is multiple myeloma (14, 16), which has been reported as having a high expression of miR-1 [50].

The loss of function of a miRNA could be due to several mechanisms; generally their genes are located in genomic regions which are unstable in cancer "cancer-associated genomic regions" (CAGRs), including genomic deletion, mutation, epigenetic silencing, and/or miRNA processing alterations [42]. Specifically, miR-1 has been found methylated in hepatocellular carcinoma [33], as well as in prostate cancer [51] and colorectal cancer [52]. miR-1 suppression in lung cancer cells is also mediated by histone hypoacetylation [47]. With respect to miR-145, this has been found methylated in colorectal cancer [53] and prostate cancer [54]. Other mechanisms have also been described that directly affect its expression, such as the p53-mediated transcriptional activation, suggesting that tumors with a low level of expression of p53, as in GBC [53, 55], have lower levels of this miRNA. Moreover, miR-145 has its coding sequence (chromosome 5q32) close to a fragile site of the genome (5q31.1) in some myelodysplastic syndromes [56] and to loss of heterozygosity or deletion of 5q in GBC [57, 58]. To date there are only two studies of microRNAs in GBC, one of which found that miR-155 is significantly overexpressed in GBC compared with normal gallbladders and this was associated with a worse prognosis; *in vitro* assays showed that miR-155 significantly enhanced cell proliferation and invasion [28].

Here, we confirmed that miR-1 and miR-145 inhibited gallbladder cancer cell growth *in vitro* by decreasing cell viability and the inhibition of colony formation or clonogenic survival. Nevertheless, this effect was higher in cells transfected with miR-1. Different studies have reported that ectopic expression of miR-1 inhibits cell growth in cancers such as prostate cancer [59], bladder cancer [60], colorectal cancer [52], hepatocellular carcinoma [33], rhabdomyosarcoma [48], lung cancer [47], squamous cell carcinoma of the maxillary sinus [61], head and neck squamous cell carcinoma (HNSCC) [62], squamous cell carcinoma of the laryngeal [63] and thyroid cancer [64]. In relation to miR-145, other studies indicate that it inhibits cell growth in gliomas [65], oral squamous cell carcinoma [66], liver cancer [35, 67], lung cancer non-small cell [68], meningioma [47] and colorectal cancer [69].

miR-1 is a known pro-apoptotic agent, the expression of which is increased during myocardial infarction in animal models [70] by regulating HSP60, HSP70 and caspase 9 [71] in rat cardiomyocytes incubated with H_2O_2 [72] and treated with a high concentration of glucose [73], inducing apoptosis. In cancer, it has been reported to induce apoptosis through caspase 3, caspase 7 and PARP-1 in lung cancer cells [47], in maxillary sinus squamous cells [61], head and neck carcinoma cell carcinomas [62], bladder cancer [60] and renal cell carcinoma

[74]. In addition, miR-145 overexpression has also been reported as inducing apoptosis in oral squamous cell carcinoma [66], hepatocarcinoma [35] and acute myeloid leukemia [75]. It has also been observed that mir-145 inhibits MDM2 mRNA, inducing apoptosis in cells of the head and neck carcinomas previously treated with cisplatin (interestingly this effect was not observed in untreated cells) [76]. It has also been seen that mir-145 induces high levels of pro-apoptotic proteins such as caspase 3, caspase 9 and PARP in glioma cells [65]. Consistent with those reports by other researchers, our group also found that miR-1 and miR-145 overexpression induces apoptosis in NOZ cells. Nevertheless, this effect was higher in cells transfected with miR-145.

As for cell cycle distribution, this study found there was no difference between the study groups. This result differs from that reported by other researchers, who have observed that both miroRNAs can alter the normal course of the cell cycle. miR-1 induces G0/G1 arrest in lung cancer [47], head and neck squamous cell carcinoma [62], renal carcinoma [74] and rhabdomyosarcoma [48]. Cell cycle arrest in G2 stage hepatocellular carcinoma and G2 arrest in HCC cells [33] has also been reported. Novello et al. observed a transient arrest (48 hours after transfection) of cell cycle in the G1 phase osteosarcoma cells transfected with miR-1, which was not detected at 72 hours [77]. In our work we found that over 50% of cells were in G/M, no differences were found with the control group. This analysis was performed at 72 hrs post-transfection; therefore, an evaluation of the cell cycle at another time might have ruled out a transient cell cycle arrest. In relation to miR-145, it is reported that it produces cell cycle arrest in G0/G1 in glioblastomas [65] and oral squamous cell carcinoma in phase G2/M in hepatocarcinoma cells with increased cyclin B1 and cyclin B2 [35] and prostate cancer [54], and in G1/S cell lines from head and neck carcinoma, esophageal cancer and lung cancer [76] and non-small cell lung cancer [68].

Also, we found that the overexpression of miR-1 significantly inhibited gene expression of VEGF-A and AXL; thus, the functional effect generated (apoptosis induction, inhibiting cell viability and formation colony) could be due to the translational repression of these targets. VEGF-A has shown remarkable overexpression in numerous human tumors, including gallbladder cancer [78]. We confirmed by an immunohistochemical study that VEGF-A is highly expressed in gallbladder cancer and correlates with poor prognosis [91]. VEGF-A is a dimeric glycoprotein essential for many angiogenic processes in normal and abnormal states, such as tumor vascularization, mainly by interacting with three tyrosine kinase receptors, VEGF-R1 (also known as Flt-1), VEGF-R2 (KDR/Flk-1) and-VEGF-R3 (Flt-4) and two membrane proteins Neuropilins (NRP1 and NRP2) [79]. The interaction of VEGF-A with VEGFR-2 activates proliferation, migration, survival and inhibition of apoptosis, mainly via the PI3K/AKT/eNOS signaling pathway through interaction with caspase 9, Bad, p38 MAPK, caspase-3 [80]. Moreover, AXL is a transmembrane receptor of 100 to 140 kDa containing an extracellular domain (N-terminal) and one intracellular tyrosine kinase (C-terminal) [81]. AXL activates phosphoinositide 3-kinase (PI3K) pathway, phospholipase C (PLC) and growth factor receptor-bound protein 2 (Grb2). The Axl/PI3K pathway inhibits apoptosis in cells through several mechanisms, particularly Akt active to S6K and BCL2, an anti-apoptotic factor. It also inhibits the pro-apoptotic effect of caspase 3 and the phosphorylate enhancer nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which also increases the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL [82]. Interestingly, the AXL signaling pathway can inhibit vascular endothelial growth factor receptor 2 (VEGFR-2) in endothelial cells by activating SHP-2 [83], so there is probably an interrelationship of these pathways through miR-1. However, the underlying mechanisms responsible for the phenotypic changes mediated by these microRNAs could occur by the regulation of multiple targets involved in complex signaling pathways. To date, a cohort of genes related to different cancer pathways have been identified and validated as targeted genes of miR-1, such as MET FOXP1, HDAC4 and PIM1 in lung cancer [47], LIM and LASP1 in bladder cancer [84]; CCND2, chemokine receptor 4 (CXCR4) and chemokine ligand 12 (CXCL12) in thyroid cancer [64]; TAGLN2 and PNP in maxillary sinus squamous cell carcinoma [61], PTMA in nasopharyngeal carcinoma [85]; fibronectin 1 in laryngeal squamous carcinoma [63], and

SRSF9 in bladder cancer [86]. On the other hand, miR-145 causes cell growth inhibition by regulating important oncogenes such as c-Myc [68], ADD3 and Sox9 in gliomas [65]. SOCS7 in bladder cancer lowers the levels of p-Akt and suppresses cell growth *in vitro*; it also promotes nuclear translocation of STAT3 [87], IRS1 and IRS2 in hepatocellular carcinoma [35], ß-catenin [88], YES and STAT1 in colon cancer [89], all genes related to cell proliferation and invasion. miR-145 also represses proliferation and promotes differentiation in pluripotency factors such as OCT4, SOX2 and KLF4 [90].

Accumulating evidence indicates that miR-1 and miR-145 are tumor suppressors involved in a wide range of neoplasms and this might also be the case for gallbladder cancer. The ectopic expression of miR-1 and miR-145, could therefore be a therapeutic strategy in the treatment of this complex and refractory disease, which must be validated by assays *in vivo*.

miRNAs have emerged as an important family of molecules with promising prospects in therapy. However, the efficacy and safety should be carefully evaluated, since the response depends on the cellular context in terms of the genetic and epigenetic profile specific to each individual. All of these approaches are still at an early stage, but with the development of new technologies, new algorithms and a more complete experimental stage, it will be used as a plausible therapeutic tool in the treatment of many diseases, including cancer.

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Disclosure of conflict of interest

None.

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