FEBS Letters 589 (2015) 2257–2265

FEBS Letters

journal homepage: www.FEBSLetters.org

miR-1275: A single microRNA that targets the three IGF2-mRNA-binding proteins hindering tumor growth in hepatocellular carcinoma

Injie Omar Fawzy [a], Mohammed Tarif Hamza [b], Karim Adel Hosny [c], Gamal Esmat [c], Hend Mohamed El Tayebi [a], Ahmed Ihab Abdelaziz [a,*]

[a] The Molecular Pathology Research Group, Department of Pharmacology, German University in Cairo, Main Entrance Al Tagamoa Al Khames, 11835 Cairo, Egypt
[b] Department of Clinical Pathology, Ain Shams University, Khalifa El-Maamoun St., Abbasiya Square, 11566 Cairo, Egypt
[c] Department of Endemic Medicine and Hepatology, Cairo University, Kasr El-Aini St., 11562 Cairo, Egypt

ARTICLE INFO

Article history:
Received 7 June 2015
Revised 24 June 2015
Accepted 26 June 2015
Available online 6 July 2015

Edited by Tamas Dalmay

Keywords:
Insulin-like growth factor-2-mRNA-binding protein (IGF2BP or IMP)
miRNA or miR
miR-1275
Hepatocellular carcinoma
Posttranscriptional regulation
Insulin-like growth factor 1 receptor (IGF1R)

ABSTRACT

This study aimed to identify a single miRNA or miR (microRNA) which regulates the three insulin-like growth factor-2-mRNA-binding proteins (IGF2BP1, 2 and 3). Bioinformatics predicted miR-1275 to simultaneously target the three IGF2BPs, and screening revealed miR-1275 to be underexpressed in hepatocellular carcinoma (HCC) tissues. Transfection of HuH-7 cells with miR-1275 suppressed IGF2BPs expression and all three IGF2BPs were confirmed as targets of miR-1275. Ectopic expression of miR-1275 and knockdown of IGF2BPs inhibited malignant cell behaviors, and also reduced IGF1R protein and mRNA. Finally IGF1R was validated as a direct target of miR-1275. These findings indicate that the tumor-suppressor miR-1275 can control HCC tumor growth partially through simultaneously regulating the oncogenic IGF2BPs and IGF1R.

1. Introduction

The pathogenesis of hepatocellular carcinoma (HCC) is multifaceted and complex, involving irregularities in several important cell signaling pathways including the insulin-like growth factor (IGF) axis. This pathway is activated when IGF-1 or IGF-2 bind to the IGF-1 receptor (IGF1R), and leads to a multitude of effects that modulate cell growth, differentiation, proliferation, and apoptosis [1].

The bioavailability of IGF-2, and hence activation of the IGF axis, can be post-transcriptionally fine-tuned by IGF-2-mRNA-binding proteins (IGF2BPs) 1, 2 and 3 [2,3]. The IGF2BPs associate with newly transcribed target mRNAs forming stable cytoplasmic complexes which have been suggested to enhance [4] or inhibit [2] translation of the mRNA, prevent the premature degradation of the mRNA by limiting its release or protecting it from the decaying actions of endonucleases or microRNAs [5–8], or assist in the long-distance transport and storage of target transcripts, especially under stress conditions [9]. These oncofetal cytoplasmic mRNA-binding proteins have been implicated in several oncogenic events in many cancers [10]. They are re-expressed in various tumors, including HCC, where their high expression is correlated with tumor invasion, early recurrence, and poor prognosis [11–15]. Moreover, IGF2BPs may play a detrimental role in malignant transformation of the liver as they have been reported to induce lipogenesis and steatohepatitis [16] and promote non-alcoholic fatty liver disease, which are well-recognized risk factors for HCC [17]. In addition, the IGF2BPs have been linked to cancer at the

Abbreviations: HCC, hepatocellular carcinoma; IGF, insulin-like growth factor; IGF1R, insulin-like growth 1 receptor; IGFBPs, insulin-like growth binding proteins; IGF2BPs or IMPs, insulin-like growth-2-mRNA-binding proteins; miRNA or miR, microRNA; HCV, hepatitis C virus; HBV, hepatitis B virus; MELD, Model for End-stage Liver Disease; PT-INR, international normalized ratio for prothrombin time; B2M, beta-2 microglobulin

Author contributions: I.O.F. contributed in study concept and design, execution of experiments, acquisition of data, statistical analysis and interpretation of data, and writing of the manuscript. M.T.H. performed and analyzed results of flow cytometry experiments and contributed in critical revision of the manuscript. K.A.H. and G.E. provided tissue samples and clinical data. H.M.E. contributed reagents. A.I.A. contributed in study concept and design and study supervision and provided critical revision of the manuscript for important intellectual content.

* Corresponding author at: The Molecular Pathology Research Group, Department of Pharmacology, German University in Cairo, New Cairo City, Main Entrance Al Tagamoa Al Khames, 11835 Cairo, Egypt. Fax: +20 2 27581041.
E-mail address: ahmed.abdel-aziz@guc.edu.eg (A.I. Abdelaziz).

http://dx.doi.org/10.1016/j.febslet.2015.06.038
0014-5793/

Table 1
Characteristic features and clinical parameters of study-recruited non-metastatic hepatocellular carcinoma (HCC) patients and healthy liver donors.

HCC and cirrhotic patients	
Mean age	49 ± 13.5
Sex: male/female	21/1
History of alcohol abuse	None
Aspartate aminotransferase (U/L)	100.5 ± 65.8
Alanine aminotransferase (U/L)	85.6 ± 95.6
Alkaline phosphatase (U/L)	110.2 ± 60.7
Serum albumin (g/dL)	4.6 ± 1.5
Serum alpha-fetoprotein (ng/mL)	155.7 ± 22.3
HCV status[a]	100% (22/22 HCC patients)
HBV status[b]	18.2% (4/22 HCC patients)
Healthy controls (liver donors)	
Mean age	31 ± 10.5
Sex: male/female	13/6
History of alcohol abuse	None
Diabetic	None
Hypertensive	None
HCV status[a]	None
HBV status[b]	None

[a] HCV status was determined using anti-HCV antibody and/or HCV viral RNA quantification.
[b] HBV status was determined using anti-HBc and anti-HBs antibodies and by detection of HBsAg.

molecular level in several in vivo and in vitro studies, where they were reported to control the post-transcriptional fate of numerous oncogenic transcripts such as *IGF-2*, *MYC*, *KRAS*, and *MKI67* [12,14,18–20].

Studies regarding the regulation of the oncofetal IGF2BPs by microRNAs – a class of small endogenous non-coding RNA molecules known to post-transcriptionally control gene expression [21] – are scarce. In fact, to date, to the best of our knowledge only miR-494 [22], the microRNAs of the let-7 family [23], and recently miR-625 [24] have been shown to regulate *IGF2BP1*, and only let-7b [25] and recently miR-216b [26] are known to target *IGF2BP2*, while the regulation of *IGF2BP3* by microRNAs has not been published as of yet. MicroRNAs are often viewed as master regulators of the human genome, and since a single microRNA can potentially control the expression of multiple genes, these short oligonucleotide sequences have quickly been recognized for their therapeutic value and have already entered clinical trials (https://clinicaltrials.gov/ct2/show/NCT01829971) [27,28].

Therefore the current study sought to identify a single microRNA which simultaneously targets all three oncogenic *IGF2BP*s, and evaluate its consequent impact on tumor growth in HCC. Through bioinformatics prediction software, gain- and loss-of-function analyses using microRNA mimics and inhibitors, and luciferase reporter assays we determined that miR-1275 is a key regulator of hepatocarcinogenesis by targeting several oncogenic members of the IGF axis.

2. Materials and methods

2.1. Patients

HCC and cirrhotic tissues from 22 HCC patients and normal liver tissues from 19 donors were obtained, with prior consent, during liver transplantation surgery at the Kasr El-Aini Hospital in Cairo, Egypt. Table 1 shows the clinical data of all study-recruited individuals. Notably, 100% of HCC patients tested positive for the presence of hepatitis C virus (HCV) infection. The assessment of liver disease in each patient is shown in Table 2. The study was approved by the appropriate ethical committees and all experiments were in accordance to the ethical standards of the declaration of Helsinki.

2.2. Bioinformatics

Bioinformatics algorithms microrna.org (http://www.microrna.org), DIANA Lab (http://www.diana.cslab.ece.ntua.gr/), and Target Scan (http://www.targetscan.org/) were used to predict microRNAs which can simultaneously target the 3′untranslated regions (3′UTRs) of *IGF2BP1*, *IGF2BP2*, and *IGF2BP3*. These algorithms were also used to predict whether miR-1275 may bind to the 3′UTR of *IGF1R*.

2.3. Cell culture and oligonucleotide transfection

HuH-7 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin/mycozap (Lonza, Switzerland). Cells were transfected with mimics or inhibitors of miR-1275, or

Table 2
Assessment of liver disease of each study-recruited non-metastatic hepatocellular carcinoma (HCC) patient.

Patients	Number of focal lesions	Size of focal lesions	MELD[a] score	Child-pugh score	PT-INR[b]
Patient 1	3 focal lesions	1.5 cm, 1 cm, 1 cm	11	C	1.4
Patient 2	Unifocal	2.5 cm	15	C_{10}	1.6
Patient 3	3 focal lesions	2 cm, 2.5 cm, 3 cm	10	C_{11}	2.4
Patient 4	3 focal lesions	2 cm, 2 cm, 3.5 cm	10	C	2
Patient 5	Unifocal	1.5 × 2 cm	16	C	1.4
Patient 6	3 focal lesions	3 × 4 cm, 1 cm, 1 cm	12	B	1.5
Patient 7	Unifocal	4 cm	11	B_7	1.5
Patient 8	3 focal lesions	4 cm, 1 cm, 1 cm	8	A_5	1.3
Patient 9	3 focal lesions	1 cm, 1 cm, 1.5 cm	17	C	1.5
Patient 10	Unifocal	2.5 cm	10	B_8	1.4
Patient 11	2 focal lesions	1 cm, 1.7 cm	15	B_8	1.4
Patient 12	3 focal lesions	1 cm, 1 cm, 1 cm	17	C_{11}	1.8
Patient 13	Unifocal	3 cm	13	B_7	1.6
Patient 14	3 focal lesions	3 cm, 1.5 cm, 2 cm	15	B	1.6
Patient 15	3 focal lesions	1 cm, 1 cm, 4 cm	12	B_7	1.14
Patient 16	2 focal lesions	3 cm, 1.5 cm	19	C_{12}	1.9
Patient 17	2 focal lesions	1.5 cm, 3 cm	16	C_{10}	1.55
Patient 18	3 focal lesions	2.5 cm, 2.5 cm, 1.5 cm	9	B_7	1.18
Patient 19	3 focal lesions	1.5 cm, 1 cm, 1 cm	11	B_8	1.19
Patient 20	Unifocal	2 cm	18	C	1.7
Patient 21	Unifocal	1.5 cm	17	C	1.55
Patient 22	3 focal lesions	3 cm, 2.5 cm, 1 cm	12	B	1.6

[a] MELD: Model for End-stage Liver Disease score.
[b] PT-INR: international normalized ratio for prothrombin time.

Table 3
Forward (F) and reverse (R) primer sequences for wild-type and mutant 3′UTR constructs.

IGF2BP1 Wild-type 3′UTR	F 5′-CAGCTTTTTTT**TCCCCCA**TAAATAAT-3′ R 5′-CTAGATTATTTA**TGGGGGA**AAAAAAAGCTGAGCT-3′
IGF2BP1 Mutant 3′UTR	F 5′-CAGCTTTTTTTAAATAAT-3′ R 5′-CTAGATTATTTAAAAAAAGCTGAGCT-3′
IGF2BP2 Wild-type 3′UTR	F 5′-CTCTACGC**ACCCCCCCCCCCCCA**GGCAAAGT-3′ R 5′-CTAGACTTTGCC**TGGGGGGGGGGGG**GTGCGTAGAGAGCT-3′
IGF2BP2 Mutant 3′UTR	F 5′-CTCTACGCACGGCAAAGT-3′ R 5′-CTAGACTTTGCCGTGCGTAGAGAGCT-3′
IGF2BP3 Wild-type 3′UTR	F 5′-CAATTCTTCTTAAT**ATTCCCCC**ATAATGCCT-3′ R 5′-CTAGAGGCATTAT**GGGGGAAT**ATTAAGAAGAATTGAGCT-3′
IGF2BP3 Mutant 3′UTR	F 5′-CAATTCTTCTTAATATAATGCCT-3′ R 5′-CTAGAGGCATTATATTAAGAAGAATTGAGCT-3′
IGF1R Wild-type 3′UTR	F 5′-CTTTTTGGGTTTTTT**TTCCCCCA**AACATTTT-3′ R 5′-CTAGAAAATGTT**TGGGGGAA**AAAAAACCCAAAAAGAGCT-3′
IGF1R Mutant 3′UTR	F 5′-CTTTTTGGGTTTTTTAACATTTT-3′ R 5′-CTAGAAAATGTTAAAAAAACCCAAAAAGAGCT-3′

siRNAs against IGF2BP1, IGF2BP2, or IGF2BP3 (Qiagen, Germany) using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer's protocol. Mock cells are cells exposed to transfection reagent only.

2.4. microRNA and mRNA extraction and quantification from liver tissues and HuH-7 cell line

Total mRNA and microRNAs were extracted from liver specimens using mirVana microRNA Isolation Kit (Ambion, USA), according to the manufacturer's protocol. HuH-7 cells were harvested using BIOZOL RNA Extraction Reagent (Bioer Technology, China). Complementary DNA was generated using reverse transcription (Applied Biosystems, USA). Relative expression of miR-1275 was normalized to RNU6B in each sample, while *IGF2BP1*, *IGF2BP2*, *IGF2BP3*, and *IGF1R* relative expressions were normalized to beta-2 microglobulin (*B2M*), and quantified using TaqMan Real-time quantitative polymerase chain reaction (RTqPCR) (Applied Biosystems). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.5. BrdU cell proliferation assay

Forty-eight hours after transfection of HuH-7 cells, proliferation was assessed according to manufacturer's protocol using the BrdU Cell Proliferation ELISA kit (Roche Applied Biosystems, Germany) and luminescence, expressed in relative light units per second, was measured.

2.6. MTT viability assay

Forty-eight hours post-transfection of HuH-7 cells 4,5-dimethyl thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) was added.

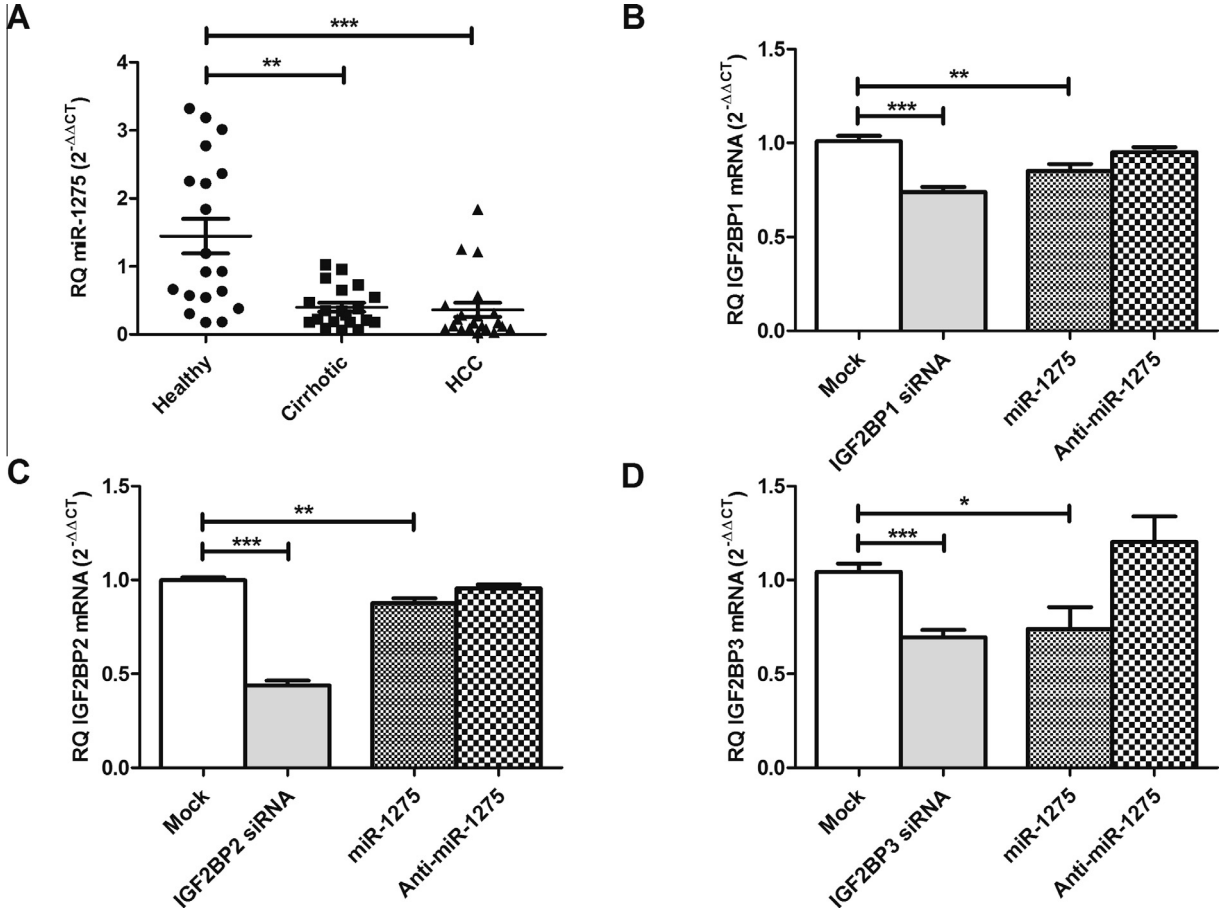

Fig. 1. Expression profile of miR-1275 in liver tissues and impact of miR-1275 on IGF2BP1, IGF2BP2, and IGF2BP3 mRNA expression in HuH-7 cell line (A) The expression of miR-1275 was investigated in healthy, cirrhotic, and HCC liver tissues using RTqPCR. Mann Whitney test was performed to compare each two groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. HuH-7 cells were transfected with miR-1275 mimics or inhibitors, and the relative expression of (B) IGF2BP1, (C) IGF2BP2, and (D) IGF2BP3 mRNA was determined using RTqPCR, relative to mock untransfected cells. Data are expressed as the mean ± SEM. Student's *t* test was performed to compare each two groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

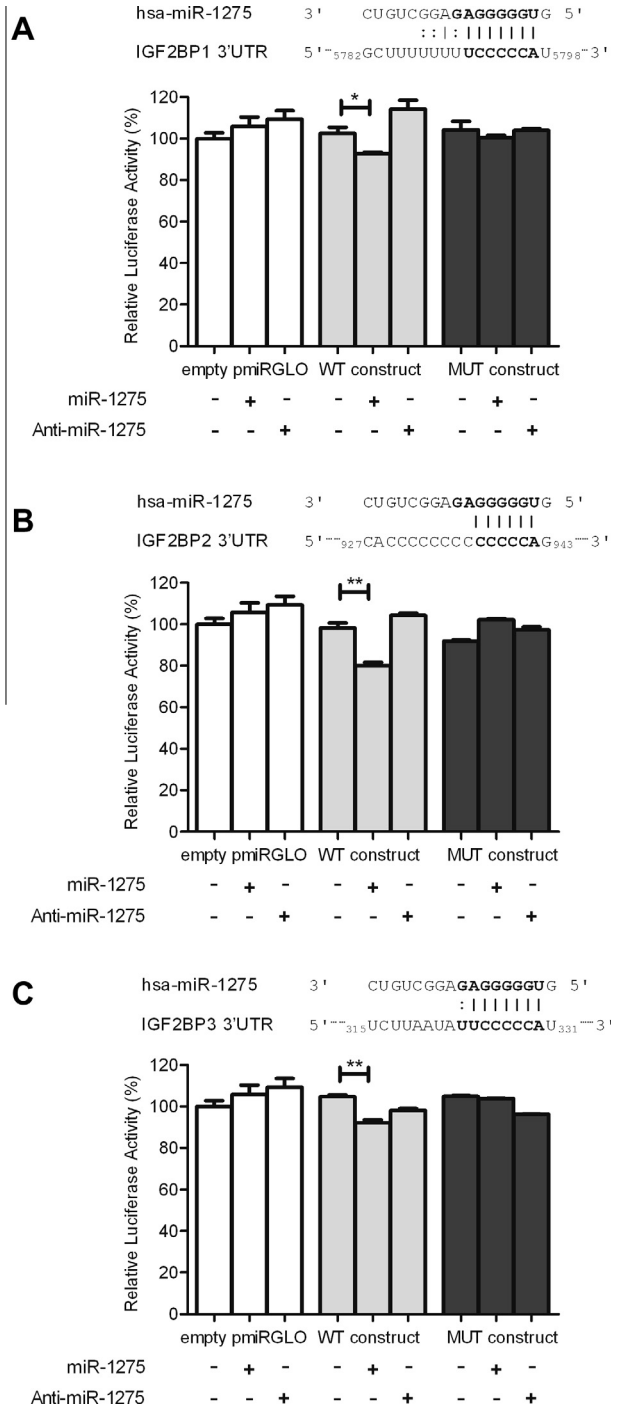

Fig. 2. Luciferase reporter assays to validate the targeting of IGF2BP1, IGF2BP2, and IGF2BP3 3′UTRs by miR-1275. For each target gene, HuH-7 cells were co-transfected with miR-1275 mimics or inhibitors and either empty pmiRGLO vector, or the wild-type (WT) construct, or the mutant (MUT) construct for (A) IGF2BP1, (B) IGF2BP2, and (C) IGF2BP3. Schematic illustration of sequence alignments between 3′UTRs of the target genes and seed-sequence of miR-1275 are shown above each corresponding graph. Data are expressed as the mean ± SEM. Student's *t* test was performed to compare each two groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

The cells were harvested and incubated with ethanol/DMSO solution and optical density was measured at 550 nm and relative viability was calculated.

2.7. Colony-forming assay

HuH-7 cells were seeded at 1000 cells/well, then transfected with oligonucleotides. Twenty-four hours post-transfection, cells were detached and imbedded in a top layer of 0.36% soft agarose overlaid on a 0.76% agarose bottom layer. The 6-well plates were incubated for two weeks to colonize.

2.8. Wound-healing assay

Forty-eight hours post-transfection of HuH-7 cells, 5 scratches/well were made, then cells were then incubated with medium supplemented with antibiotic and 1% FBS for 24 h. Randomly chosen fields were photographed under a microscope (AXIO observer A1, ZEISS, Germany) at 10X magnification and areas of scratches were recorded at time 0 h and 24 h and percentages of wound closures were calculated.

2.9. Reporter constructs and Luciferase assay

For wild-type constructs (WT), the miR-1275 binding regions in the 3′UTRs of the longest isoforms of *IGF2BP1*, *IGF2BP2*, *IGF2BP3*, or *IGF1R* were inserted in the pmirGLO Dual-Luciferase microRNA Target Expression Vector (Promega, USA). In addition, mutant constructs (MUT) were designed, as shown in Table 3; the sequences in bold indicate the binding region nucleotides which were deleted to form the mutant constructs. HuH-7 cells were transfected with 2 μg of the constructs or empty pmirGLO vector using SuperFect (Qiagen) then co-transfected 24 h later with miR-1275 mimics or inhibitors using HiPerFect (Qiagen). Relative luciferase activity was measured after 48 h by the Luciferase Reporter Assay Kit (Biovision, USA).

2.10. Flow cytometric analysis

At 72 h post-transfection cell aliquots were incubated with primary antibody (IGF1Rα Antibody (1H7), Santa Cruz Biotechnology, USA) for 20 min followed by washing with PBS. Cells were incubated with secondary antibody (goat anti-mouse IgG-FITC, Santa Cruz Biotechnology) for 20 min in the dark, then washed and resuspended in PBS, and analyzed immediately using a Coulter Epics XL flow cytometer (Beckman-coulter, Florida, USA). The mean fluorescence signal intensity (MFI) was analyzed using WinMDI software (http://facs.scripps.edu/soft-ware.htm) supplied by Dr. Joe Trotter, Salk Institute (La Jolla, CA, USA) and calculated as the percentage of that of mock cells.

2.11. Statistical analysis

All experiments were performed in triplicates and repeated at least three times. The data were expressed as the mean ± standard error of the mean (SEM). All analyses, unless otherwise stated, were performed using GraphPad Prism version 5 and a two-tailed value of $P < 0.05$ was considered statistically significant with Student's *t*-test.

3. Results

3.1. Expression of miR-1275 is downregulated in cirrhotic and cancerous liver tissues

Bioinformatics was used to identify microRNAs which could simultaneously target *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* transcripts,

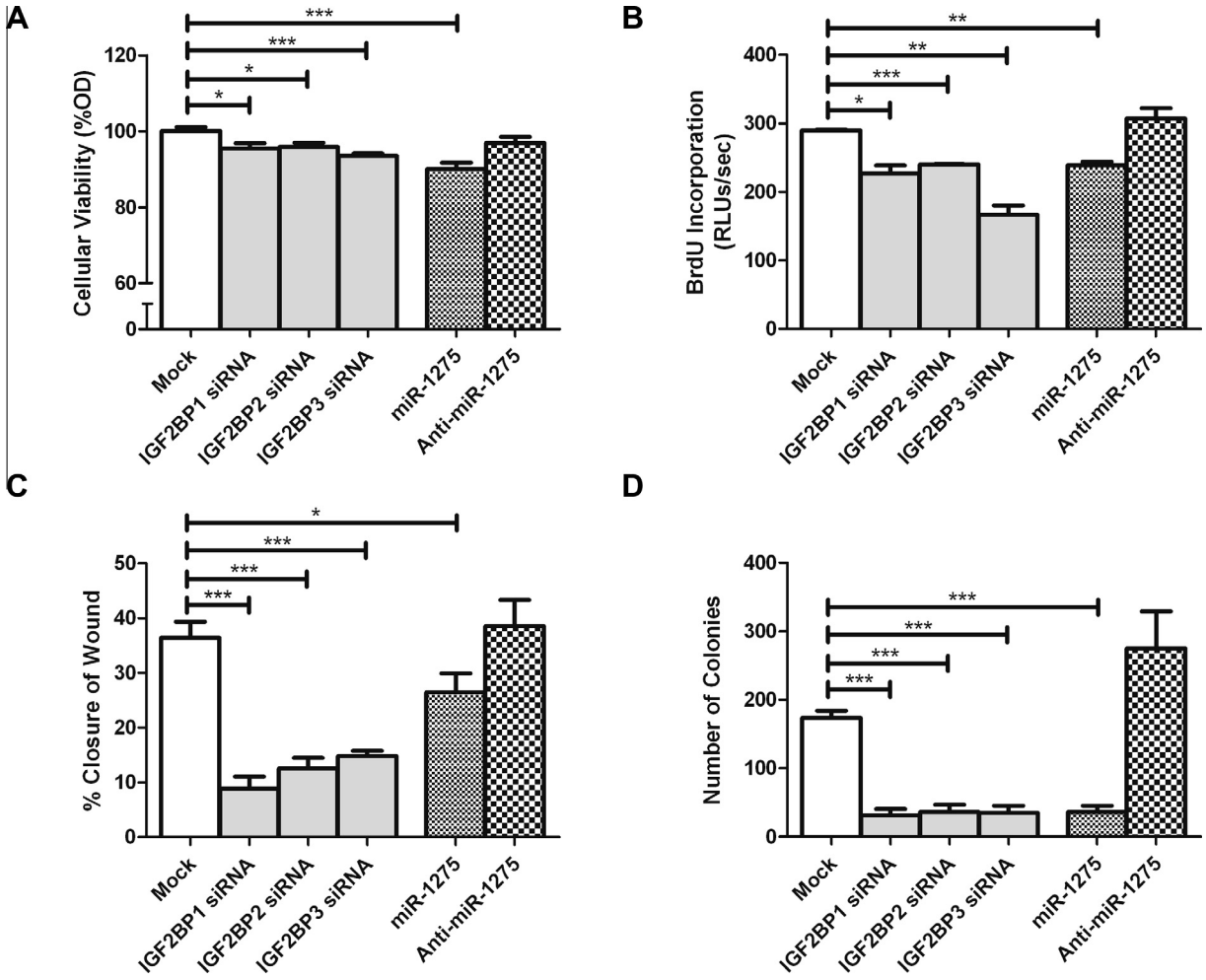

Fig. 3. Impact of miR-1275 on viability, proliferation, migration, and clonogenicity of HuH-7 cell line. HuH-7 cells were transfected with miR-1275 mimics or inhibitors. Also, oncofetal IGF2BP1, IGF2BP2, and IGF2BP3 were silenced in HuH-7 cells using specific siRNAs. In all experiments, data are expressed as the mean ± SEM and Student's *t* test was performed to compare each two groups, where $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. (A) MTT assay for cellular viability, (B) BrdU incorporation assay for cellular proliferation, (C) wound-healing assay for tumor cell migration, (D) colony-forming assay for clonogenicity were performed.

and miR-1275 was selected as a candidate for further testing. To investigate the expression of miR-1275 in liver cancer, RTqPCR was performed in 22 HCC and cirrhotic tissues and 19 healthy liver tissues. Screening revealed that both cirrhotic ($P = 0.0013$) and HCC ($P < 0.0001$) tissues of HCC patients have markedly lower expression of miR-1275 than healthy liver tissues from liver donors suggesting that miR-1275 becomes underexpressed during the malignant transformation of liver tissue (Fig. 1A).

3.2. Overexpression of miR-1275 decreases IGF2BP1, IGF2BP2, and IGF2BP3 mRNA expression in HuH-7 cell line

Gain- and loss-of-function experiments were performed to determine the effect of miR-1275 on the IGF2BPs. Ectopic expression of miR-1275 resulted in a noticeable decrease in *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* mRNA levels compared to mock cells ($P = 0.0037$, $P = 0.0014$, and $P = 0.0119$, respectively) (Fig. 1B–D). On the other hand, inhibition of miR-1275 showed *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* expression levels comparable to those observed in mock cells. Nonetheless these findings demonstrate that, similar to siRNAs against *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* ($P < 0.0001$, $P < 0.0001$, and $P = 0.0001$, respectively), miR-1275 can dramatically diminish the expression of the *IGF2BP*s (Fig. 1).

3.3. IGF2BP1, IGF2BP2, and IGF2BP3 are direct targets of miR-1275

To confirm that miR-1275 directly targets *IGF2BP1*, *IGF2BP2*, and *IGF2BP3*, the binding region from the 3′UTR of each target gene was inserted downstream to the luciferase reporter gene in pmiRGLO vector. A mutant construct for each binding site was also prepared in which the predicted binding sequence had been deleted. In a set of cells, empty pmiRGLO vector was also transfected as a control to ensure that miR-1275 oligonucleotides have no effect on the vector itself. For IGF2BP1, IGF2BP2, and IGF2BP3 WT constructs, luciferase activity was markedly inhibited when cells were co-transfected with miR-1275 mimics ($P = 0.0313$, $P = 0.0035$, and $P = 0.0021$, respectively) (Fig. 2A–C, respectively), indicating direct targeting and transcriptional inhibition of the target genes by miR-1275. On the other hand, luciferase activity showed no change when WT constructs were co-transfected with miR-1275 inhibitors. Moreover, the effect was abolished when the

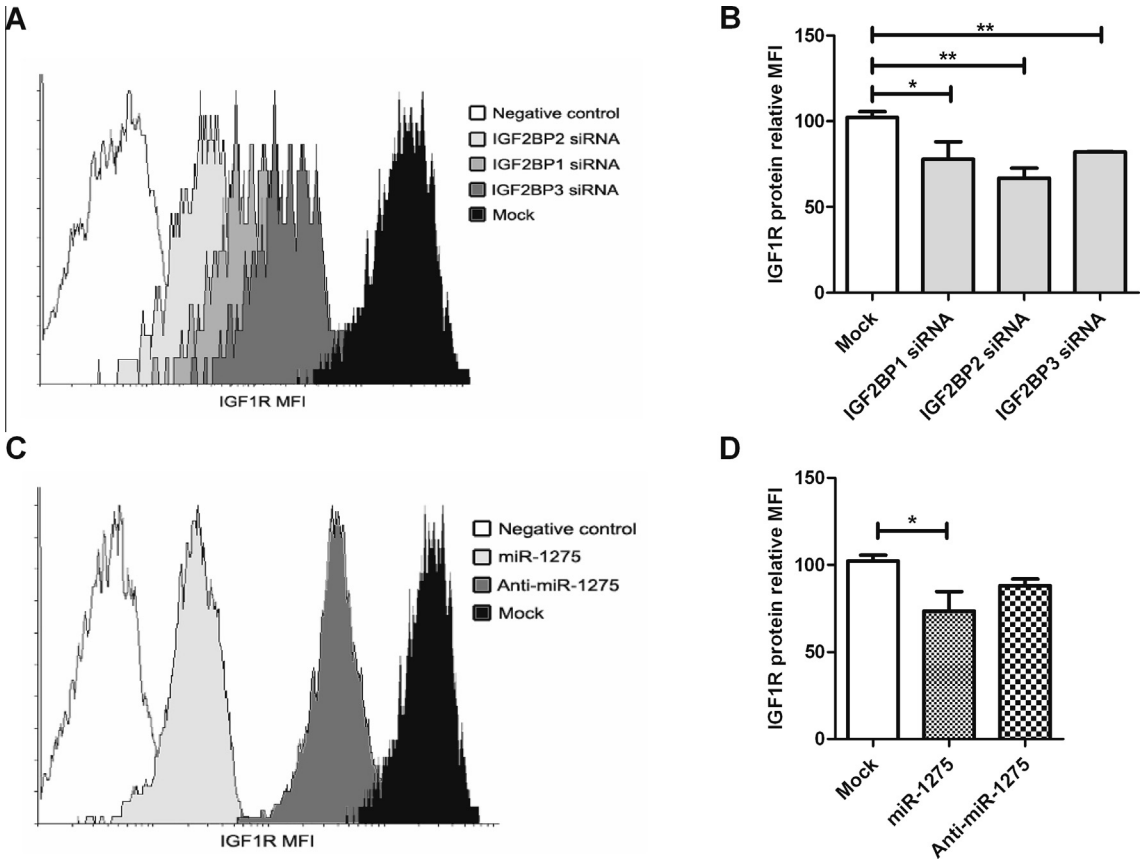

Fig. 4. Impact of IGF2BP knockdown and miR-1275 on IGF1R protein expression in HuH-7 cell line. (A) HuH-7 cells were transfected with specific siRNAs against each of the three IGF2BPs and the level of IGF1R protein was evaluated using flow cytometry. (B) The mean fluorescence intensity (MFI) of knockdown cells compared to mock cells as measured by flow cytometry. (C) HuH-7 cells were transfected with miR-1275 mimics or inhibitors and the level of IGF1R protein was evaluated using flow cytometry. (D) The MFI of cells transfected with miR-1275 mimics or inhibitors was compared to mock cells as measured by flow cytometry. All data are expressed as the mean ± SEM. Student's *t* test was performed to compare each two groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

nucleotides in seed binding sites of the 3′UTRs were deleted (Fig. 2). These findings assert the necessity of the binding region nucleotides for the efficient targeting of these genes by miR-1275.

3.4. Overexpression of miR-1275 inhibits viability, proliferation, wound-healing, and clonogenicity of HuH-7 cell line

Since the role of miR-1275 in hepatocarcinogenesis has never been explored, MTT, BrdU, migration, and colony-formation assays were performed to evaluate its overall effect on various characteristic properties of tumor cells. MiR-1275 or siRNAs against IGF2BPs were transiently transfected in HuH-7 cells. Compared to mock cells, ectopic expression of miR-1275 significantly suppressed cellular viability ($P < 0.0001$), proliferation ($P = 0.0038$), wound-healing ($P = 0.0362$), and clonogenicity ($P = 0.0005$) (Fig. 3A–D). As expected, siRNAs against *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* also drastically decreased the viability ($P = 0.0256$, $P = 0.0364$, and $P = 0.0007$, respectively), proliferation ($P = 0.0266$, $P < 0.0001$, and $P = 0.0056$, respectively), wound-healing ($P < 0.0001$, $P = 0.0003$, and $P = 0.0004$, respectively), and clonogenicity ($P = 0.0005$, $P = 0.0008$, and $P = 0.0007$, respectively) of HuH-7 cells compared to mock cells (Fig. 3). In contrast, cells transfected with miR-1275 inhibitors showed properties similar to those of mock cells (Fig. 3). Taken together, these results emphasize the impact of miR-1275 on hepatocarcinogenesis through its effect on the *IGF2BP*s.

3.5. IGF2BP knockdown and miR-1275 overexpression decrease IGF1R protein expression in HuH-7 cell line

Previous studies have indicated that the IGF2BPs play a role in regulating *IGF-2* transcripts [2–4], but whether the IGF2BPs can control the post-transcriptional fate of the *IGF1R* mRNA in humans has not been investigated. Flow cytometric analysis revealed that silencing of *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* in HuH-7 cells all significantly reduced IGF1R protein levels compared to mock cells ($P = 0.0405$, $P = 0.0012$, and $P = 0.0043$, respectively) (Fig. 4A and B). Since ectopic expression of miR-1275 suppressed the *IGF2BP*s, it was intriguing to discover whether this microRNA would have similar effects on IGF1R. Indeed, forcing the expression of miR-1275 caused a considerable decrease in IGF1R protein compared to mock cells ($P = 0.05$) (Fig. 4C and D).

3.6. IGF2BP knockdown and miR-1275 overexpression decrease IGF1R transcript levels in HuH-7 cell line

Interestingly, transfection of siRNAs against *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* all led to a marked decrease in *IGF1R* mRNA levels compared to mock cells ($P < 0.0001$, $P < 0.0001$, and $P = 0.0064$, respectively) (Fig. 5A). Transfection of miR-1275 mimics also markedly decreased *IGF1R* mRNA levels compared to mock cells ($P = 0.0023$). On the other hand, cells transfected with miR-1275 inhibitors showed *IGF1R* transcript levels similar to mock cells (Fig. 5B). The observation revealed that miR-1275 attenuated the

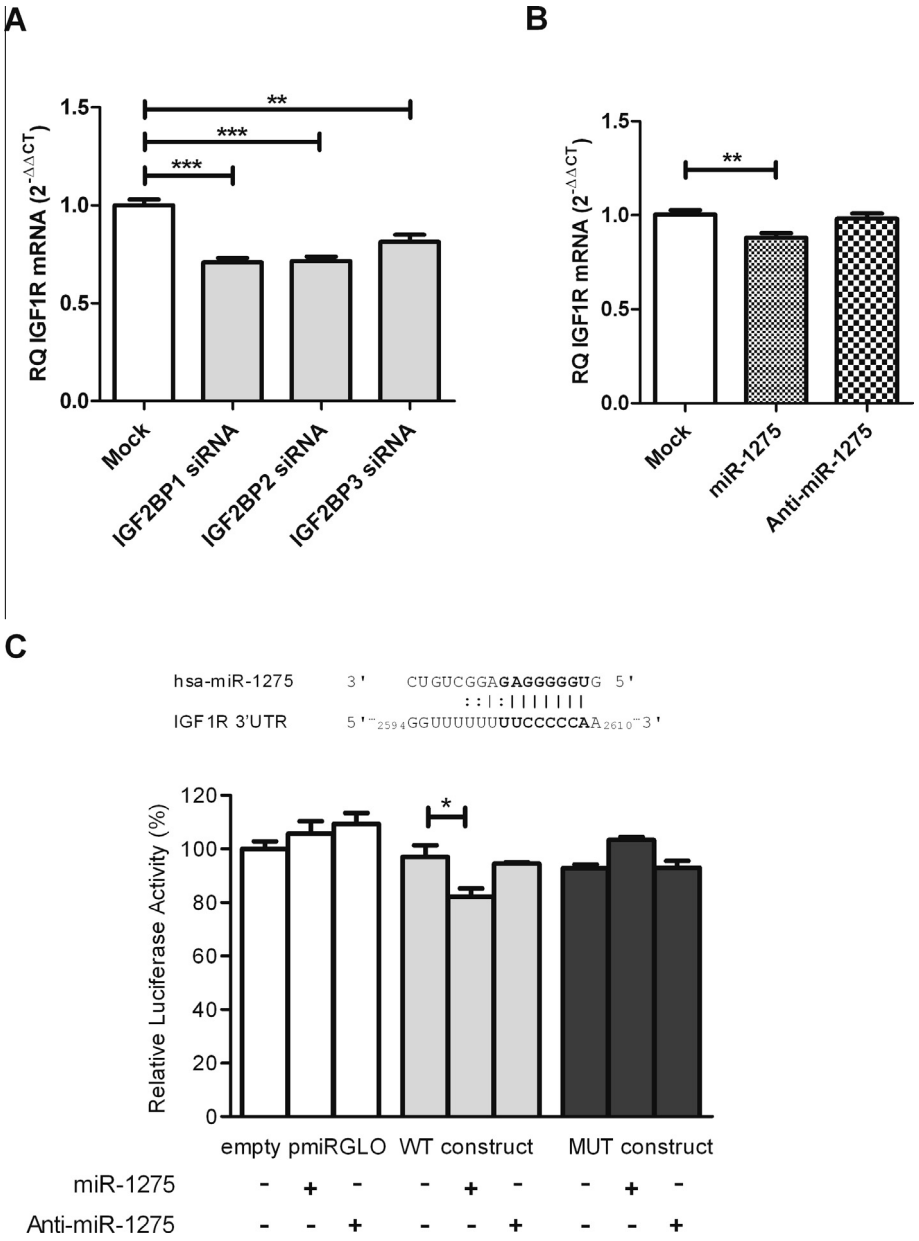

Fig. 5. Impact of IGF2BP knockdown and miR-1275 on IGF1R mRNA expression in HuH-7 cell line and validation of IGF1R as a direct target of miR-1275. (A) The relative expression of IGF1R mRNA was determined using RTqPCR in HuH-7 cells transfected with specific siRNAs against each of the IGF2BPs relative to mock cells. (B) The relative expression of IGF1R mRNA was determined in cells transfected with miR-1275 mimics or inhibitors relative to mock cells. (C) Luciferase reporter assay was used to validate the targeting of IGF1R 3′UTR by miR-1275. HuH-7 cells were co-transfected with miR-1275 mimics or inhibitors and either empty pmiRGLO vector, or the wild-type (WT) IGF1R 3′UTR construct, or the mutant (MUT) IGF1R 3′UTR construct. Schematic illustration of sequence alignment between 3′UTR of the IGF1R and seed-sequence of miR-1275 is shown above the graph. Data are expressed as the mean ± SEM. Student's *t* test was performed to compare each two groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

expression of IGF1R via or partly by decreasing the expression of the IGF2BPs, thus suppressing the viability, proliferation, migration, and clonogenicity of HuH-7 cells.

3.7. IGF1R is a direct target of miR-1275

Since a single microRNA can have multiple targets, it was important to examine whether miR-1275 may also have a direct regulatory effect on *IGF1R* mRNA. In silico analysis search results yielded that miR-1275 is in fact predicted to target the *IGF1R* 3′UTR. The binding region from the 3′UTR of *IGF1R* was inserted downstream to luciferase reporter gene in pmiRGLO vector to form the IGF1R WT construct. A mutant construct was also prepared in which the predicted binding region had been deleted. For IGF1R WT construct, luciferase activity was markedly inhibited when cells were co-transfected with miR-1275 mimics ($P = 0.0497$, respectively) (Fig. 5C), while no change was observed upon co-transfection with miR-1275 inhibitors, or when the MUT construct was co-transfected with miR-1275 mimics or inhibitors (Fig. 5C). These findings indicate that miR-1275 directly binds to the 3′UTR of *IGF1R*.

4. Discussion

The dysregulation of microRNAs occurs frequently in many cancers including HCC, and, as post-transcriptional regulators of gene expression, these small non-coding RNAs have been implicated in cancer formation and progression [29]. The interest in microRNAs

as a therapeutic strategy stems from the ability of these endogenous molecules to simultaneously control several aberrantly expressed genes allowing for a multi-target therapy from a single molecule. The IGF2BPs are a family of three highly homologous mRNA-binding proteins which lock their target transcripts into a specific conformation and control their transcriptional fate [30]. Their repertoire of target transcripts includes numerous cancer-implicated genes and for this reason they present a promising approach for controlling cancer development [12,14,18–20]. By bioinformatic analysis we predicted miR-1275 to simultaneously target *IGF2BP1*, *IGF2BP2*, and *IGF2BP3*, a desirable property which may bestow upon this microRNA the ability to indirectly regulate many oncogenic transcripts through the control of these mRNA-binding proteins. We found that miR-1275 is severely underexpressed in HCC and cirrhotic liver from patients with HCV-induced liver cancer. A previous study also identified miR-1275 as a downregulated microRNA in HBV-associated HCC [31]. This may imply that the underexpression of miR-1275 occurs in malignant transformation of the liver regardless of the etiology.

Surprisingly few papers have reported the regulation of the IGF2BPs by microRNAs, none of which have identified a single microRNA which can simultaneously target all members of the family. The present study, however, demonstrates that ectopic expression of miR-1275 concomitantly reduces *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* mRNA. Using microRNA-target expression constructs it was demonstrated that all three *IGF2BP*s are the direct targets of miR-1275.

Several lines of evidence implicate the IGF2BPs in cancer development. An IGF2BP1-knockout mouse model displayed dwarfism, reduced viability, decreased cellular proliferation, and increased apoptosis [32], while IGF2BP1 overexpression in adult female transgenic mice led to very high incidence of tumors which were generally multifocal, and several tumor-bearing mice had metastases [33]. In vitro depletion of IGF2BP1 from multiple liver cancer cell lines inhibited proliferation and induced apoptosis, which was attributed to its ability to stabilize the *c-MYC* and *MKI67* mRNAs and increase c-Myc and Ki-67 protein expression, two potent regulators of cell proliferation and apoptosis [12]. Moreover, a murine xenograft model with stably-depleted IGF2BP1 showed impaired tumor growth [12]. The liver-specific overexpression of *p62*, a splice variant of IGF2BP2 in which exon 10 is skipped, in mice was demonstrated to strongly induce *Igf2* expression [34]; similarly, in human hepatoma HepG2 cell lines, overexpression of *p62* led to increased *IGF-2* and antiapoptotic effects [35]. Also, depletion of IGF2BP3 in HCC cell line HA22T caused a decrease in cell motility, invasion, and transendothelial migration [13]. Consistent with these other studies, we found that knockdown of each of the three IGF2BPs severely impairs HuH-7 cell viability, proliferation, migration, and colony-formation. Moreover, correcting the expression of the dysregulated miR-1275 which suppresses the IGF2BPs also led to similar effects. Due to its underexpression in certain cancers, miR-1275 has previously been referred to as a tumor-suppressor [31,36], however this is the sole study to perform comprehensive functional analysis experiments and portray the net tumor suppressive effects of this microRNA in HCC.

Although many studies have focused on the role of IGF2BPs in regulating *IGF-2* transcripts, few studies have considered whether IGF2BPs can influence the transcriptional fate of another crucial member of the IGF axis, the *IGF1R*. A previous study overexpressed the IGF2BPs and identified thousands of possible binding mRNAs, and of note, *IGF1R* transcript was among the top 100 binding mRNAs [37]. However, to date, the effects the IGF2BPs may have upon binding to *IGF1R* mRNA in humans is unknown.

Findings of this study show for the first time that knockdown of *IGF2BP1*, *2*, or *3* extensively reduces IGF1R protein, suggesting that all three IGF2BPs act to enhance the protein levels of this oncogenic receptor. Since IGF2BPs have been known to increase protein levels of their targets either by enhancing translation or by preventing degradation of the transcript, it was essential to also assess their effects on the *IGF1R* mRNA. This study demonstrates that silencing of each IGF2BP additionally leads to a decrease in *IGF1R* transcript levels. Taken together with the previous RNA-immunoprecipitation study [37], this insinuates that IGF2BPs may act to stabilize the *IGF1R* mRNA and/or shield it to prevent its decay, as they have been reported to do for several other target transcripts including *CD44*, *CTNNB1*, and *KRAS* [19,38,39]. Interestingly, however, these findings are in partial disagreement with a study conducted on murine myoblasts, which found that knockdown of *IGF2BP2* reduced IGF1R protein yet did not significantly change the level of *Igf1r* mRNA [40]. The effect of an IGF2BP on its target transcript may be dependent on its phosphorylation status, the recruitment of other regulatory proteins to the complex, or *cis*-determinants in the target transcript [4,10,41]. Therefore the incongruities observed between the two studies may be attributable to species or cell-type differences, or to several aforementioned factors which may influence the function of the IGF2BPs.

We found that similar to the effects of knocking down the IGF2BPs, forcing the expression of miR-1275 also results in a considerable decrease in IGF1R mRNA and protein. Additionally, in silico analysis revealed a binding region for miR-1275 in the 3′UTR of the *IGF1R* transcript and luciferase activity assays demonstrated that *IGF1R* is a direct target of miR-1275. Hence, it can be construed that miR-1275 not only directly targets the *IGF1R*, but may also indirectly modulate IGF1R levels by controlling its post-transcriptional regulators, the *IGF2BP*s.

In conclusion, we found that miR-1275 is downregulated in cirrhotic and cancerous liver tissues, and forcing its expression suppresses the *IGF2BP*s and effectively impairs tumor cell proliferation, migration, viability, and colony-formation. *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* were validated as direct targets for this microRNA. Moreover we showed that knockdown of the *IGF2BP*s decreases IGF1R mRNA and protein, and that *IGF1R* 3′UTR is targeted by miR-1275. Taken together, we demonstrated for the first time that, miR-1275 inhibits malignant cell behaviors by negatively regulating multiple oncogenic members of the IGF axis. Thus both miR-1275 and its target genes, the IGF2BPs and the IGF1R, present viable therapeutic targets for liver cancer.

Acknowledgements

The study was supported by the Egyptian Science and Technology Development Fund (STDF) Grant 4242. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] Wu, J. and Zhu, A.X. (2011) Targeting insulin-like growth factor axis in hepatocellular carcinoma. J. Hematol. Oncol. 4, 30.
[2] Nielsen, J., Christiansen, J., Lykke-Andersen, J., Johnsen, A.H., Wewer, U.M. and Nielsen, F.C. (1999) A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. Mol. Cell. Biol. 19, 1262–1270.
[3] Liao, B., Hu, Y. and Brewer, G. (2011) RNA-binding protein insulin-like growth factor mRNA-binding protein 3 (IMP-3) promotes cell survival via insulin-like growth factor II signaling after ionizing radiation. J. Biol. Chem. 286, 31145–31152.
[4] Dai, N., Rapley, J., Angel, M., Yanik, M.F., Blower, M.D. and Avruch, J. (2011) MTOR phosphorylates IMP2 to promote IGF2 mRNA translation by internal ribosomal entry. Genes Dev. 25, 1159–1172.
[5] Lemm, I. and Ross, J. (2002) Regulation of c-myc mRNA decay by translational pausing in a coding region instability determinant. Mol. Cell. Biol. 22, 3959–3969.
[6] Noubissi, F.K., Elcheva, I., Bhatia, N., Shakoori, A., Ougolkov, A., Liu, J., Minamoto, T., Ross, J., Fuchs, S.Y. and Spiegelman, V.S. (2006) CRD-BP

mediates stabilization of betaTrCP1 and c-myc mRNA in response to beta-catenin signalling. Nature 441, 898–901.
[7] Sparanese, D. and Lee, C.H. (2007) CRD-BP shields c-myc and MDR-1 RNA from endonucleolytic attack by a mammalian endoribonuclease. Nucleic Acids Res. 35, 1209–1221.
[8] Elcheva, I., Goswami, S., Noubissi, F.K. and Spiegelman, V.S. (2009) CRD-BP protects the coding region of betaTrCP1 mRNA from miR-183-mediated degradation. Mol. Cell 35, 240–246.
[9] Stohr, N., Lederer, M., Reinke, C., Meyer, S., Hatzfeld, M., Singer, R.H. and Huttelmaier, S. (2006) ZBP1 regulates mRNA stability during cellular stress. J. Cell Biol. 175, 527–534.
[10] Bell, J.L., Wachter, K., Muhleck, B., Pazaitis, N., Kohn, M., Lederer, M. and Huttelmaier, S. (2013) Insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs): post-transcriptional drivers of cancer progression? Cell. Mol. Life Sci. 70, 2657–2675.
[11] Yaniv, K. and Yisraeli, J.K. (2002) The involvement of a conserved family of RNA binding proteins in embryonic development and carcinogenesis. Gene 287, 49–54.
[12] Gutschner, T., Hammerle, M., Pazaitis, N., Bley, N., Fiskin, E., Uckelmann, H., Heim, A., Grobeta, M., Hofmann, N., Geffers, R., Skawran, B., Longerich, T., Breuhahn, K., Schirmacher, P., Muhleck, B., Huttelmaier, S. and Diederichs, S. (2014) Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) is an important protumorigenic factor in hepatocellular carcinoma. Hepatology 59, 1900–1911.
[13] Jeng, Y.M., Chang, C.C., Hu, F.C., Chou, H.Y., Kao, H.L., Wang, T.H. and Hsu, H.C. (2008) RNA-binding protein insulin-like growth factor II mRNA-binding protein 3 expression promotes tumor invasion and predicts early recurrence and poor prognosis in hepatocellular carcinoma. Hepatology 48, 1118–1127.
[14] Wachter, D.L., Kristiansen, G., Soll, C., Hellerbrand, C., Breuhahn, K., Fritzsche, F., Agaimy, A., Hartmann, A. and Riener, M.O. (2012) Insulin-like growth factor II mRNA-binding protein 3 (IMP3) expression in hepatocellular carcinoma. A clinicopathological analysis with emphasis on diagnostic value. Histopathology 60, 278–286.
[15] Chen, L.T., Lin, L.J. and Zheng, L.L. (2013) The correlation between insulin-like growth factor II mRNA binding protein 3 expression in hepatocellular carcinoma and prognosis. Hepatogastroenterology 60, 553–556.
[16] Laggai, S., Kessler, S.M., Boettcher, S., Lebrun, V., Gemperlein, K., Lederer, E., Leclercq, I.A., Mueller, R., Hartmann, R.W., Haybaeck, J. and Kiemer, A.K. (2014) The IGF2 mRNA binding protein p62/IGF2BP2-2 induces fatty acid elongation as a critical feature of steatosis. J. Lipid Res. 55, 1087–1097.
[17] Simon, Y., Kessler, S.M., Bohle, R.M., Haybaeck, J. and Kiemer, A.K. (2014) The insulin-like growth factor 2 (IGF2) mRNA-binding protein p62/IGF2BP2-2 as a promoter of NAFLD and HCC? Gut 63, 861–863.
[18] Noubissi, F.K., Nikiforov, M.A., Colburn, N. and Spiegelman, V.S. (2010) Transcriptional regulation of CRD-BP by c-myc: implications for c-myc functions. Genes Cancer 1, 1074–1082.
[19] Mongroo, P.S., Noubissi, F.K., Cuatrecasas, M., Kalabis, J., King, C.E., Johnstone, C.N., Bowser, M.J., Castells, A., Spiegelman, V.S. and Rustgi, A.K. (2011) IMP-1 displays cross-talk with K-Ras and modulates colon cancer cell survival through the novel proapoptotic protein CYFIP2. Cancer Res. 71, 2172–2182.
[20] Suvasini, R., Shruti, B., Thota, B., Shinde, S.V., Friedmann-Morvinski, D., Nawaz, Z., Prasanna, K.V., Thennarasu, K., Hegde, A.S., Arivazhagan, A., Chandramouli, B.A., Santosh, V. and Somasundaram, K. (2011) Insulin growth factor-2 binding protein 3 (IGF2BP3) is a glioblastoma-specific marker that activates phosphatidylinositol 3-kinase/mitogen-activated protein kinase (PI3K/MAPK) pathways by modulating IGF-2. J. Biol. Chem. 286, 25882–25890.
[21] Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. Science 294, 853–858.
[22] Ohdaira, H., Sekiguchi, M., Miyata, K. and Yoshida, K. (2012) MicroRNA-494 suppresses cell proliferation and induces senescence in A549 lung cancer cells. Cell Prolif. 45, 32–38.
[23] Cheng, M., Si, Y., Niu, Y., Liu, X., Li, X., Zhao, J., Jin, Q. and Yang, W. (2013) High-throughput profiling of alpha interferon- and interleukin-28B-regulated microRNAs and identification of let-7s with anti-hepatitis C virus activity by targeting IGF2BP1. J. Virol. 87, 9707–9718.
[24] Zhou, X., Zhang, C.Z., Lu, S.X., Chen, G.G., Li, L.Z., Liu, L.L., Yi, C., Fu, J., Hu, W., Wen, J.M. and Yun, J.P. (2015) MiR-625 suppresses tumour migration and invasion by targeting IGF2BP1 in hepatocellular carcinoma. Oncogene 34, 965–977.
[25] Alajez, N.M., Shi, W., Wong, D., Lenarduzzi, M., Waldron, J., Weinreb, I. and Liu, F.F. (2012) Lin28b promotes head and neck cancer progression via modulation of the insulin-like growth factor survival pathway. Oncotarget 3, 1641–1652.
[26] Liu, F.Y., Zhou, S.J., Deng, Y.L., Zhang, Z.Y., Zhang, E.L., Wu, Z.B., Huang, Z.Y. and Chen, X.P. (2015) MiR-216b is involved in pathogenesis and progression of hepatocellular carcinoma through HBx-miR-216b-IGF2BP2 signaling pathway. Cell Death Dis. 6, e1670.
[27] Janssen, H.L., Reesink, H.W., Lawitz, E.J., Zeuzem, S., Rodriguez-Torres, M., Patel, K., van der Meer, A.J., Patick, A.K., Chen, A., Zhou, Y., Persson, R., King, B.D., Kauppinen, S., Levin, A.A. and Hodges, M.R. (2013) Treatment of HCV infection by targeting microRNA. N. Engl. J. Med. 368, 1685–1694.
[28] Bader, A.G. (2012) MiR-34 – a microRNA replacement therapy is headed to the clinic. Front. Genet. 3, 120.
[29] Callegari, E., Elamin, B.K., Sabbioni, S., Gramantieri, L. and Negrini, M. (2013) Role of microRNAs in hepatocellular carcinoma: a clinical perspective. OncoTargets Ther. 6, 1167–1178.
[30] Nielsen, J., Kristensen, M.A., Willemoes, M., Nielsen, F.C. and Christiansen, J. (2004) Sequential dimerization of human zipcode-binding protein IMP1 on RNA: a cooperative mechanism providing RNP stability. Nucleic Acids Res. 32, 4368–4376.
[31] Wang, W., Zhao, L.J., Tan, Y.X., Ren, H. and Qi, Z.T. (2012) Identification of deregulated miRNAs and their targets in hepatitis B virus-associated hepatocellular carcinoma. World J. Gastroenterol. 18, 5442–5453.
[32] Hansen, T.V., Hammer, N.A., Nielsen, J., Madsen, M., Dalbaeck, C., Wewer, U.M., Christiansen, J. and Nielsen, F.C. (2004) Dwarfism and impaired gut development in insulin-like growth factor II mRNA-binding protein 1-deficient mice. Mol. Cell. Biol. 24, 4448–4464.
[33] Tessier, C.R., Doyle, G.A., Clark, B.A., Pitot, H.C. and Ross, J. (2004) Mammary tumor induction in transgenic mice expressing an RNA-binding protein. Cancer Res. 64, 209–214.
[34] Tybl, E., Shi, F.D., Kessler, S.M., Tierling, S., Walter, J., Bohle, R.M., Wieland, S., Zhang, J., Tan, E.M. and Kiemer, A.K. (2011) Overexpression of the IGF2-mRNA binding protein p62 in transgenic mice induces a steatotic phenotype. J. Hepatol. 54, 994–1001.
[35] Kessler, S.M., Pokorny, J., Zimmer, V., Laggai, S., Lammert, F., Bohle, R.M. and Kiemer, A.K. (2013) IGF2 mRNA binding protein p62/IMP2-2 in hepatocellular carcinoma: antiapoptotic action is independent of IGF2/PI3K signaling. Am. J. Physiol. Gastrointest. Liver Physiol. 304, G328–G336.
[36] Kahlert, C., Klupp, F., Brand, K., Lasitschka, F., Diederichs, S., Kirchberg, J., Rahbari, N., Dutta, S., Bork, U., Fritzmann, J., Reissfelder, C., Koch, M. and Weitz, J. (2011) Invasion front-specific expression and prognostic significance of microRNA in colorectal liver metastases. Cancer Sci. 102, 1799–1807.
[37] Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano Jr., M., Jungkamp, A.C., Munschauer, M., Ulrich, A., Wardle, G.S., Dewell, S., Zavolan, M. and Tuschl, T. (2010) Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 141, 129–141.
[38] Vikesaa, J., Hansen, T.V., Jonson, L., Borup, R., Wewer, U.M., Christiansen, J. and Nielsen, F.C. (2006) RNA-binding IMPs promote cell adhesion and invadopodia formation. EMBO J. 25, 1456–1468.
[39] Gu, W., Wells, A.L., Pan, F. and Singer, R.H. (2008) Feedback regulation between zipcode binding protein 1 and beta-catenin mRNAs in breast cancer cells. Mol. Cell. Biol. 28, 4963–4974.
[40] Li, Z., Gilbert, J.A., Zhang, Y., Zhang, M., Qiu, Q., Ramanujan, K., Shavlakadze, T., Eash, J.K., Scaramozza, A., Goddeeris, M.M., Kirsch, D.G., Campbell, K.P., Brack, A.S. and Glass, D.J. (2012) An HMGA2-IGF2BP2 axis regulates myoblast proliferation and myogenesis. Dev. Cell 23, 1176–1188.
[41] Weidensdorfer, D., Stohr, N., Baude, A., Lederer, M., Kohn, M., Schierhorn, A., Buchmeier, S., Wahle, E. and Huttelmaier, S. (2009) Control of c-myc mRNA stability by IGF2BP1-associated cytoplasmic RNPs. RNA 15, 104–115.