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miR-615-5p is restrictedly expressed in cirrhotic and cancerous liver tissues and its overexpression alleviates the tumorigenic effects in hepatocellular carcinoma

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ABSTRACT

microRNAs aberrant behavior in heptocellular carcinoma (HCC) plays a major role in HCC pathogenesis. miR-615-5p expression has never been evaluated in HCC. We showed that miR-615-5p was preferentially expressed in HCC, cirrhotic liver tissues and HCC cell lines, but undetected in normal livers. Forced miR-615-5p expression in HCC cell lines led to significant decrease in cell growth and migration. In-silico predication revealed insulin-like growth factor-II (IGF-II) as a potential downstream target for miR-615-5p. Forcing the expression of miR-615-5p showed downregulation of IGF-II mRNA, as well as inhibition of the luciferase activity in a luciferase reporter vector harboring the IGF-II-3′UTR target sequence. miR-615-5p acts as tumor-suppressor in HCC through targeting IGF-II.

1. Introduction

Complexity of the pathogenesis of hepatocellular carcinoma (HCC) evoked researches to look what's beyond the genetic disruption in the disease which is the epigenetic regulation of HCC pathogenesis and progression. Hepatocarcinogenesis is governed by many epigenetic regulatory processes like hypermethylation of tumor suppressor genes FBP1, RASSF1 and SOX17 that was evidenced frequently in HCC tissues leading to their silencing and increased cellular proliferation, cell cycle progression and nuclear accumulation of mitogenic proteins [1–3]. On the other hand, hypomethylation of oncogenes CD147 and LINE-1 in HCC that led to increased metastasis and poor survival [4,5]. Emerging evidence indicates that the newly discovered non-coding short RNAs (microRNAs) deregulation have contributed greatly to the epigenetic-based hepatocarcinogensies [6]. microRNAs (miRNA) originate from intronic or polycistronic sequences and were proved to have roles in cellular differentiation, metabolism, proliferation and malignant transformation [7]. Some microRNAs were found to be overexpressed in HCC and were classified to be oncogenic in nature as miR-155 that promoted cellular invasion and proliferation in HCC cell lines[8,9]. Moreover, miR-210 was also described as an oncomiR in promoting HCC cell invasion and migration [10]. Antagonizing the expression of miR-221 in HCC cell lines, that is clustered as oncogenic miRNA lead to reduced cellular proliferation [11]. Some other microRNAs showed potent anti-tumorgenic effects where they were mostly downregulated in HCC tissues compared to healthy liver tissues. For example, the liver specific miR-122; was found to be downregulated in HCC tissues, while its intended overexpression showed anti-proliferative effects in human HCC cell lines [12]. Also, miR-520e, miR-223 and miR-375 were downregulated in HCC tissues. Upon their intended overexpression, they showed potent proliferation-inhibition, cell cycle arrest, apoptosis and clonogenicity inhibition in HCC cell lines [13–15]. miR-615-5p is a very novel microRNA that was once analyzed in prostate cancer cell lines where it showed high expression [16]. However the role of miR-615-5p has never been studied and its role in carcinogenesis has never been investigated which makes it a potential target for further analysis. Hence our study aimed at investigating miR-615-5p in HCC in an approach to screen for the presence of miR-615-5p in healthy, cirrhotic and cancerous liver tissues as well as HCC cell lines followed by its functional analysis by inducing its overexpression and inhibition in HuH-7 and HepG2 cell lines. We have simultaneously screened and manipulated the expression of the recently characterized hepato-oncogenic miR-155 as standard control for our results.

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2. Materials and methods

2.1. Study patients

This study included 20 HCC patients who underwent liver transplant surgery in the Kasr El Einy Hospital, Cairo University, Egypt. Nine cirrhotic tissues were taken from some of the same HCC patients with focal HCC lesions. Fifteen healthy liver biopsies were obtained. The study was approved by the Cairo University's ethical review committee. All participants gave their written informed consent. Most of the patients (75.5%) had more than one focal lesion as indicated in the pathology report and were subjected to clinical assessment as shown in Table 1.

2.2. Cell cultures

HuH-7 and HepG2 cells were maintained in Dulbecco's modified Eagle's medium(DMEM) (Lonza, Switzerland) supplemented with 4.5 g/l glucose, 4 mmol/l L-glutamine, 10% fetal bovine serum and Mycozap (1:500, Lonza, Switzerland) at 37 °C in 5% CO_2 atmosphere.

2.3. Transfection of microRNA oligonucleotides

HuH-7 and HepG2 cell lines were transfected with mimics and inhibitors of miR-615-5p, miR-155 and scrambled microRNAs (Qiagen, Germany). For examining the effect of miR-615-5p on Insulin-like growth factor (IGF-II) transcript expression, siRNAs against IGF-II were used (FlexiTube Gene Solution IGF-II-specific siRNAs, Qiagen, Germany). All transfection experiments were carried out in triplicates using HiPerfect Transfection Reagent (Qiagen, Germany) according to the manufacture's protocol and experiments were repeated 3 times. Cells that were only exposed to transfection reagent were designated as Mock cells, cells transfected with scrambled microRNAs were designated as Scr-miR cells, and cells transfected with siRNAs against IGF-II were designated as siIGF-II cells. Cells transfected with miR-615-5p or miR-155 mimics were designated as miR-615-5p cells or miR-155 cells, respectively and cells transfected with the respective miR-615-5p or miR-155 inhibitor were designated as anti-miR-615-5p or anti-miR-155 cells, respectively.

2.4. mRNA and microRNA extraction from liver biopsies and HCC cell lines

mRNAs and microRNAs were extracted from liver biopsies and HCC cell lines. Fresh liver samples (HCC, cirrhotic and healthy tissues) were collected during surgery and were immediately snap-frozen in liquid nitrogen. The specimens were manually pulverized in liquid nitrogen and about 100 mg of tissues powder were used for large and small RNA extraction using mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacture's protocol. HCC cell lines were harvested 48 h after transfection according to HiPerfect Transfection Reagent protocol: 150 ng oligonucleotides were used for HuH-7 and 300 ng oligonucleotides for HepG2 cells transfection in 6-well plate. RNA yield was quantified with a spectrophotometer and RNA integrity was tested by 18S rRNA bands detection on 1% agarose gel electrophoresis. RNA samples with Optical Density 260/280 more than 2 were excluded from the study.

Table 1
Characteristic features of hepatocellular carcinoma (HCC) patients.

Age: mean	49
Sex: male/female	19/1
Aspartate aminotransferase (AST) (U/l)	100.5 ± 65.8
Alanine aminotransferase (ALT) (U/l)	85.6 ± 95.6
Alkaline Phosphatase (U/l)	110.2 ± 60.7
Serum albumin (g/dl)	4.6 ± 1.5
Serum AFP (ng/ml)	155.7 ± 22.3
HCV Ab	100% (20 HCC patients)

2.5. miRNA and mRNA quantification

The extracted microRNAs were reverse transcribed into single stranded complementary DNA (cDNA) using TaqMan® MicroRNA Reverse Transcription Kit (ABI, USA) and specific primers for hsa-miR-615-5p, hsa-miR-155 and RNU6B. IGF-II mRNA was reverse transcribed into cDNA using the High-capacity cDNA Reverse Transcription Kit (ABI, USA) according to the manufacturer's instruction. Relative expression of miR-615-5p and miR-155 and RNU6B (for normalization) as well as IGF-II and GAPDH (for normalization) was quantified using TaqMan Real-Time Q-PCR (ABI Assay IDs: 002353, 002623, 001093, Hs01005963_m1 and Hs00266705_g1, respectively) using **StepOne**™ Systems (ABI, USA). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All PCR reactions including controls were run in duplicate reactions.

2.6. Cell proliferation and growth assays (BrdU and MTT)

For BrdU assay; HuH-7 and HepG2 cells were seeded 24 h prior to transfection in black 96-well plates and transfected with 12.5 ng and 25 ng oligonucleotides, respectively (according to HiPerfect protocol) with initial constant cell count, 5×10^4 cells/well. Forty-eight hours after oligonucleotides transfection, cells were labeled with BrdU labeling reagent for 4 h (with final concentration 100 μM) using the Cell Proliferation ELISA kit (Roche Applied Biosystems, Germany). Cells were then fixed using FixDenate for 30 min then incubated with Anti-BrdU POD (with final concentration 10 μM) for 90 min. For MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay; 10,000 HuH-7 and HepG2 cells were seeded in 200 μl media per well in a 96 well plate and incubated 24 h prior to transfection with 12.5 ng and 25 ng oligonucleotides, respectively (according to HiPerfect protocol). Forty-eight hours post-transfection, 20 μl MTT solution (5 mg/ml MTT in PBS) was added to each well. After incubation for 5 h, formazan (MTT metabolic product) was resuspended in 200 μl DMSO. Colorimetric measurements and absorbance were performed using *Wallac 1420 Victor2 Multilabel Counter* (Perkin Elmer, USA). All cell proliferation experiments were done in triplicates and repeated three times.

2.7. Cell scratch wound healing assay

HuH-7 and HepG2 cells were left for 80–90% confluence in 6-well plates. Forty-eight hours post-transfection, 5 scratches/well were made in each plate with 200 μl pipette tip. Detached cells were washed out using serum-free medium. Medium was then added and culture plates were incubated at 37 °C. Forty-eight hours post-scratching, migration was documented and wound closure was quantified with *Image J* software (http://rsbweb.nih.gov/ij/download.html) by measuring the surface area covered by the cells. All Scratch assays were done in duplicates (two wells/test which represents 10 scratches/test) and repeated three times.

2.8. Bioinformatics

Using bioinformatics algorithms; microrna.org (www.microrna.org), miRDB (www.mirdb.org/miRDB/), DIANA Lab (www.diana.cslab.ece.ntua.gr/), and Target Scan (www.targetscan.org/) putative downstream targets for miR-615-5p have been predicted.

2.9. IGF-II 3′UTR construct and luciferase assay

The predicted target site for miR-615-5p at IGF-II 3′UTR was designed as oligonucleotides and flanked by SacI and XbaI restriction sites; Sense: 5′GAGCTCTAGGTACCGCAAAGAGAAAAGAAGGACCCCATCTAGA3′and antisense: 5′TCTAGATGGGGTCCTTCTTTTCTCTTTGCGGTACCATGAGCC-TC 3′. pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Germany) was double digested with SacI and XbaI restriction enzymes (Takara, Japan) at 37 °C for 4 h followed by enzymes deactivation at 65 °C, then blunted by T4 DNA polymerase enzyme (Fermentas, USA) at 11 °C for 20 min. Sense and antisense strands of the designed oligonucleoitdes were annealed at 90 °C for 3 min then incubated at 37 °C for 15 min. Annealed double stranded oligonucleotides were ligated to the pmiRGLO vector double digest using T4 DNA ligase (Fermentas, USA) at 22 °C with overnight incubation. HuH-7 cells were transfected by lipofection; Superfect transfection reagent (Qiagen, Germany) with pmiRGLO+/−insert in presence or absence of miR-615-5p mimics and inhibitors. After 48 h luciferase assay was performed using Luciferase Reporter System (Promega, Germany) by adding 100 μl 1× cell lysis reagent per well in 24 well plate followed by transferring 20 μl of cell lysate into 96 well plate. Luciferase reagent was added and chemilumenicence was measured within 10 s.

3. Statistical analysis

All data are presented as mean with SEM. Statistical significance of the data was analyzed by performing One-way analysis of variance (ANOVA) or Paired Student *t* tests. The specific types of tests, when applicable, are indicated in the figure legends. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns = statistically not significant.

4. Results

4.1. miR-615-5p and miR-155 screening in liver tissues and HCC cell lines

miR-615-5p was undetectable in 100% of healthy liver tissues. While its expression was significantly high in all HCC biopsies ($n = 20$) and cell lines (HuH-7 and HepG2) compared to cirrhotic tissues ($P = 0.007$, $P < 0.0001$ and $P = 0.0013$, respectively; Fig. 1a). miR-155 showed a significant higher expression in HCC ($n = 20$) and cirrhotic tissues ($n = 9$) as well as HCC cell lines compared to healthy tissues ($P = 0.0003$, $P = 0.0001$ and $P < 0.0001$, respectively). It also worth mentioning that miR-155 was markedly elevated in HCC tissues compared to other cirrhotic tissues ($P = 0.0001$).

4.2. Transfection efficiency of miR-615-5p and miR-155 oligonucleotides

Mimicked HuH-7 and HepG2 cells with miR-615-5p and miR-155 mimics showed upregulation up to 47.12, 61.2 ($P = 0.0025$ and $P = 0.003$, respectively), 20 and 17 ($P < 0.0001$ and $P < 0.0001$, respectively) fold increase compared to their respective mock cells (Fig. 2).

4.3. Impact of miR-615-5p and miR-155 on cellular proliferation and growth

BrdU incorporation assay was used for investigating cell proliferation and growth. Mimicking of miR-615-5p in HuH-7 and HepG2 cells showed a significant decrease in cellular proliferation ($P = 0.0002$ and $P = 0.0044$, respectively) compared to negative control and mock cells while miR-155 promoted HuH-7 and HepG2 cell proliferation significantly ($P = 0.0017$ and $P = 0.0292$, respectively). Recovery experiments using antagomirs for both miR-615-5p and miR-155 showed significant recovery of the inhibitory effect of miR-615-5p on proliferation and decrease in the proliferative effect of miR-155 on HuH-7 and HepG2 ($P < 0.0001$, $P = 0.0004$) and ($P < 0.0001$, $P < 0.0001$), respectively (Fig. 3a).

MTT assay was used for investigating cell viability and growth. Forced expression of miR-615-5p in HuH-7 and HepG2 cell lines showed marked repression in cellular growth ($P = 0.0471$ and $P = 0.0072$, respectively) compared to mock cells while miR-155 showed increased HuH-7 and HepG2 cell growth ($P = 0.0102$ and 0.0002, respectively). Recovery experiments using inhibitors for both miR-615-5p and miR-155 showed reversal of the inhibitory effect of miR-615-5p and stimulatory effect of miR-155 on HuH-7 and HepG2 cell growth ($P = 0.1185$ and $P = 0.0207$) and ($P = 0.0038$ and $P = 0.0001$), respectively (Fig. 3b).

4.4. Impact of miR-615-5p and miR-155 on cellular migration

Images for two-dimensional scratch-migration assay were documented at fivefold magnification[17]. Transfection of miR-615-5p

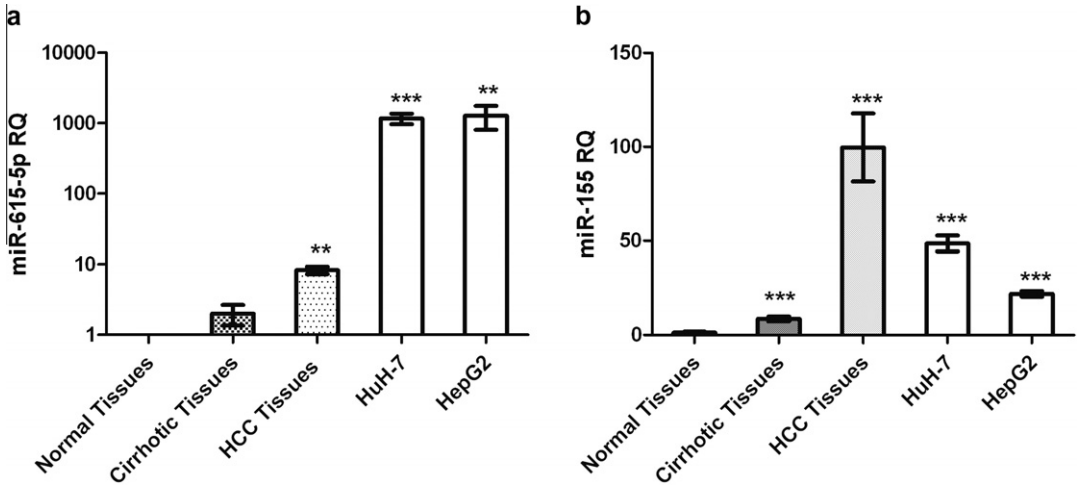


Fig. 1. miR-615-5p and miR-155 screening in liver tissues and HCC cell lines; (a) miR-615-5p expression was undetectable in healthy liver biopsies with increased expression in HCC tissues and HuH-7 and HepG2 cell lines compared to cirrhotic tissues. (b) miR-155 showed increased expression in HCC and cirrhotic tissues as well as in HuH-7 and HepG2 cell lines compared to healthy tissues. One-way analysis of variance (ANOVA) was performed.

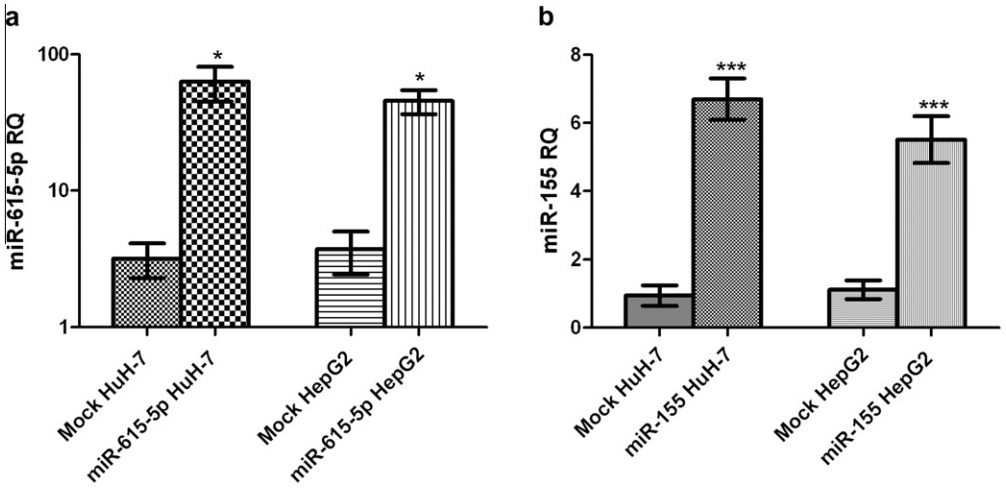


Fig. 2. Transfection efficiency of miR-615-5p and miR-155 oligonucleotides; (a) miR-615-5p mimics significantly increased its expression in HuH-7 and HepG2 cells compared to mock control. (b) miR-155 mimics elevated its expression in HuH-7 and HepG2 cells compared to mock controls. Paired *t* test was performed.

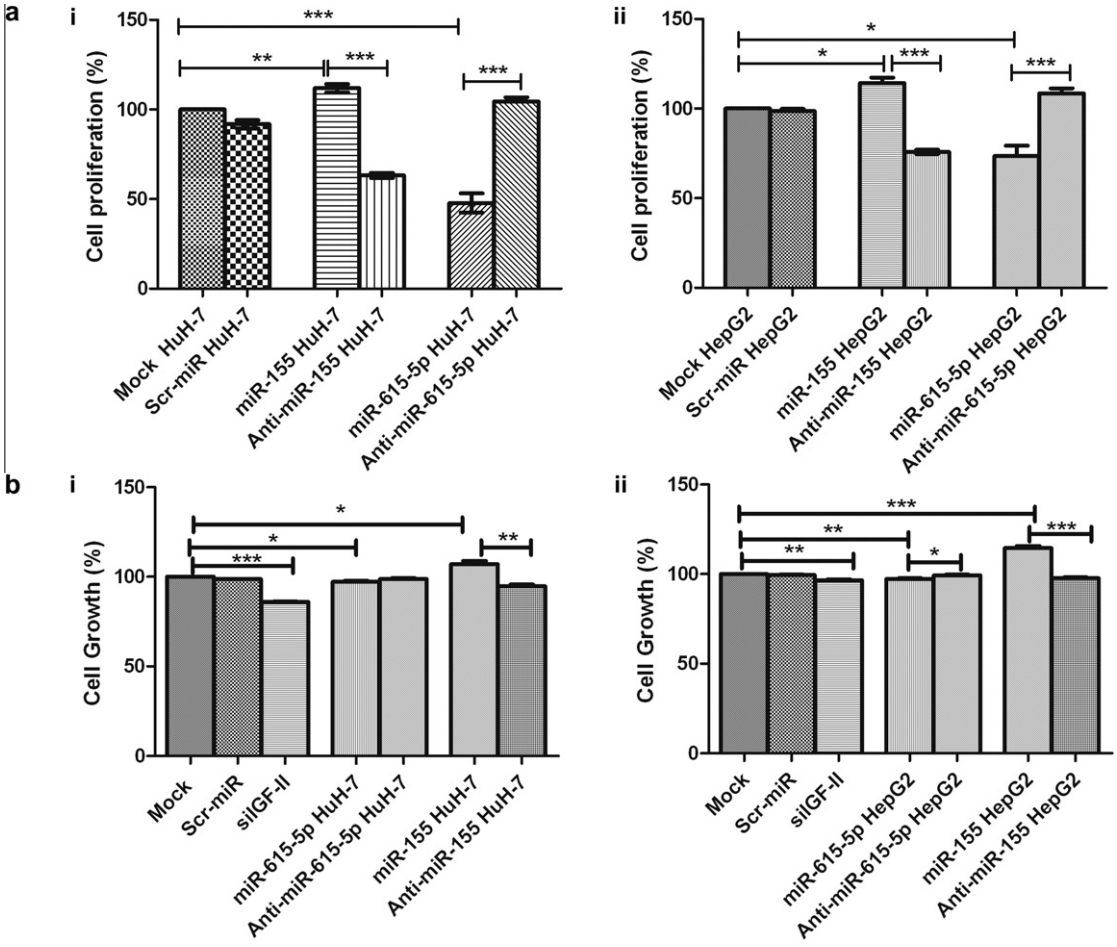


Fig. 3. Impact of miR-615-5p and miR-155 on cellular growth; (a) BrdU assay; mimics of miR-615-5p significantly suppressed cellular proliferation and growth in HuH-7 and HepG2 cells compared to controls. While mimics of miR-155 markedly promoted proliferation compared to controls. Inhibitors of miR-615-5p significantly promoted cellular proliferation compared to miR-615-5p mimicked cells while inhibitors of miR-155 significantly repressed proliferation compared to miR-155 mimicked cells. (b) MTT assay; In HuH-7 and HepG2 cells, miR-615-5p mimics markedly inhibited cellular growth and viability in contrast to miR-155 that significantly increased cellular growth compared to mock cells. Inhibitors of miR-615-5p improved cellular growth compared to miR-615-5p mimicked cells while anti-miR-155 cells showed repressed growth compared to miR-155 cells. One-way analysis of variance (ANOVA) was performed for statistical analysis.

mimics led to significant reduction in tumor cell migration in HuH-7 and HepG2 cells covering 77.86% and 45.3% of the original scratch compared to mock and Scr-miR with original scratch coverage of (84.2%, 82.7%) and (100%, 100%) in both cell lines respectively. While miR-155 mimics resulted in promotion of tumor cell migration in HuH-7 and HepG2 cells covering 89% and 100% of the original scratch compared to mock and Scr-miR with original scratch coverage of (84.2%, 82.7%) and (100%, 100%) in both cell

lines respectively (Fig. 4). Migration inhibition by miR-615-5p was rescued using miR-615-5p anatgomirs in HuH-7 and HepG2 cells, while migration induction by miR-155 was repressed using miR-155 antagomirs (Fig. 4).

4.5. Bioinformatics

miR-615-5p accession number and mature sequences were retrieved using miRBase database (http://www.mirbase.org/). In silico predictions was carried out using 4 different software, IGF-II was found to be a predicted target for miR-615-5p. miR-615-5p was found to hit IGF-II at 5 different regions by the same seed sequence (Fig. 5a). Binding scores given by softwares representing the hybridization energies exerted for miR-615-5p-IGF-II binding are shown in Fig. 5b.

4.6. IGF-II transcript as a putative target of miR-615-5p

Mimicking of miR-615-5p in HuH-7 and HepG2 cells resulted in a significant downregulation of IGF-II mRNA levels ($P = 0.0001$ and $P < 0.0001$, respectively) compared to controls (Fig. 6). Inhibitors of miR-615-5p in HuH-7 and HepG2 cells resulted in a marked increase in IGF-II mRNA levels compared to miR-615-5p mimicked cells ($P < 0.0001$ and $P < 0.0001$, Fig. 6).

4.7. Correlation analysis between miR-615-5p and IGF-II mRNA expression in HCC tissues

IGF-II mRNA was quantified in all HCC tissues and correlated to miR-615-5p expression in the same patients. Using Pearson statistical method of correlation, miR-615-5p expression was found to be inversely correlated with IGF-II transcript expression in all HCC tissues studied with Pearson $r = -0.468$ ($P = 0.0371$, Fig. 7).

4.8. IGF-II is a direct target of miR-615-5p

To confirm that miR-615-5p directly targets the 3′UTR of IGF-II, 3′UTR target region was inserted downstream to luciferase reporter gene in pmiRGLO vector. Experiments were carried out by trasnfecting HuH-7 cells with either empty pmiRGLO vector or the vector with the miR-615-5p target region insert. miR-615-5p mimics and inhibitors were co-transfected with both vectors. Upon miR-615-5p mimics co-transfection, luciferase activity was inhibited by 19.4% in cells with the pmiRGLO + insert compared to cells transfected with the pmiRGLO–insert ($P < 0.0001$). Upon miR-615-5p inhibitors co-transfection, luciferase activity was increased by 9% compared to cells transfected pmiRGLO-insert and by 28% compared to pmiRGLO + insert co-transfected with miR-615-5p mimics ($P = 0.0007$). The luciferase experiments were done in triplicates.

5. Discussion

Several microRNAs have shown elevated expression in cancerous tissues compared to normal liver tissue like miRNA-135a and miR-423 that showed abundant expression in HCC compared to normal liver tissues, and were found to promote tumorgenesis by increasing the metastatic and invasive behaviors in vitro as well as promoting cell growth and cell cycle progression [17,18]. In contrast to these findings, some microRNAs have shown lower expression in HCC tissues compared to normal tissues and their intended overexpression suppressed HCC proliferation and migration greatly like miR-122 and miR-199a/b-3p [19,20]. While in a third scenario some microRNAs showed no expression in normal tissues and were re-expressed in cancerous tissues as miR-615. miR-615 was overexpressed in prostate cancer and disappeared from normal prostate epithelial cells [16]. miR-615-5p has never been investigated in HCC and it is still query whether it has protumorgenic or anti-tumorgenic effects. For the first time, we identified that miR-615-5p is expressed in 100% of the HCC tissues and cell lines as well as cirrhotic tissues but was undetectable in 100% of healthy liver tissues. In order to validate these results, we have chosen miR-155 as a positive control as it was proven to have a positive proliferative and migratory effect in HCC [8,9]. miR-155 was found to be significantly upregulated in all HCC as well as cirrhotic tissues compared to healthy liver tissues. To analyze the impact of miR-615-5p on hepatocarcinogenesies, we ectopically induced the expression of miR-615-5p in different HCC cell lines. This overexpression has markedly repressed cellular growth examined by

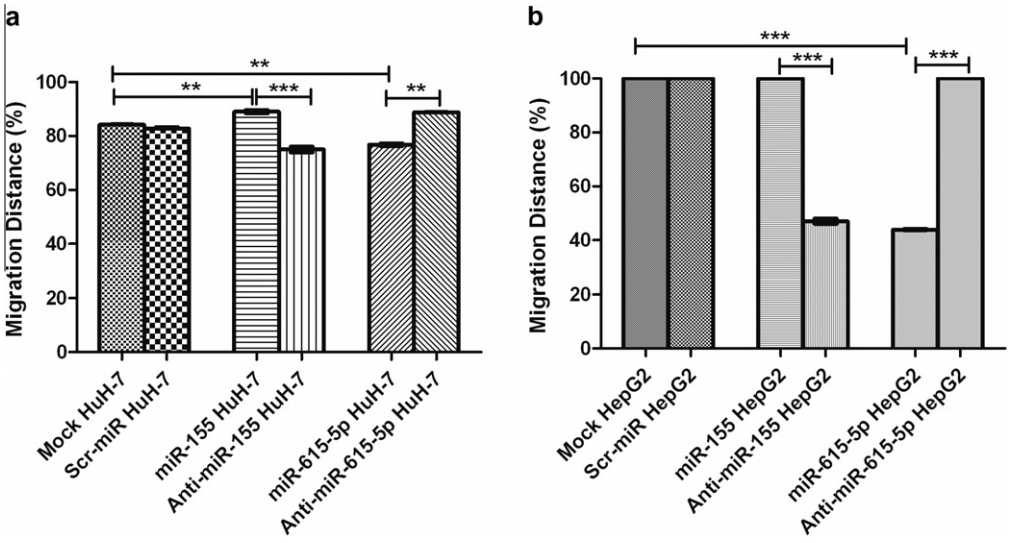
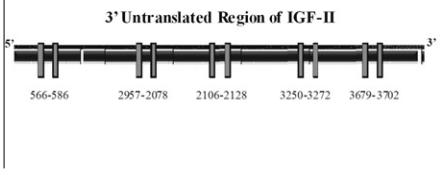


Fig. 4. Impact of miR-615-5p and miR-155 on cellular Migration; (a) miR-615-5p mimics significantly inhibited cellular migration in HuH-7 cells while miR-155 mimics markedly induced migration compared to the original scratch. Inhibitors of miR-615-5p significantly promoted cellular migration compared to miR-615-5p mimicked cells while miR-155 inhibitors suppressed migration significantly compared to miR-155 mimicked cells. (b) miR-615-5p mimics significantly inhibited cellular migration in HepG2 cells while miR-155 mimics markedly induced migration that reached complete closure of the original scratch. Inhibitors of miR-615-5p significantly promoted cellular migration with complete closure of the scratch compared to miR-615-5p mimicked cells while miR-155 inhibitors suppressed migration significantly compared to miR-155 mimicked cells. One-way analysis of variance (ANOVA) was performed for statistical analysis.

a



b

MIMAT0004804: Mature miR-615-5p sequence: GGGGGUCCCCGGUGCUCGGAUC

Softwares	Seeding Sequence (Binding site on 3'UTR of IGF -II)	Position on 3'UTR of IGF-II	Complem -entarty %	Binding Score
1.microrna.org 2.DIANA Lab 3.Target Scan 4. miRDBase	1. 3' cuaggCUCGUGGC-CCCUGGGGg 5' miR-615-5p 5'gcaaaGAGAAAAGAAGGACCCCa 3' IGF-II	566-586	54.54%	-0.5102
1.microrna.org 2.DIANA Lab 3.Target Scan 4. miRDBase	2. 3'cuaggCUCGUGGCCCCUGGGGg 5' miR-615-5p 5'aggaaGAUC-UUGGGGACCCCc 3' IGF-II	2057-2078	54.54%	-0.0055
1.microrna.org 2.DIANA Lab 3.Target Scan 4. miRDBase	3. 3' cuaGGCUCGUGGCCCCUGGGGg 5' miR-615-5p 5' ugcCCGUCUCCUGGGGACCCCc 3' IGF-II	2106-2128	59.09%	-0.0144
1.microrna.org 2.DIANA Lab	4. 3' cuaggcucguggccc CUGGGGg 5' miR-615-5p 5' ugacggggcuggcau GACCCCg 3' IGF-II	3250-3272	27.27%	-0.0001
1.microrna.org 2.DIANA Lab	5. 3' cuAGGCUCGUGG-CCCCUGGGGg 5' miR-615-5p 5' ucUGCCAUCACCUGAAGACCCCc 3' IGF-II	3679-3702	59.09%	-0.0022

Fig. 5. Bioinformatics analysis for the binding between miR-615-5p and IGF-II; (a) Schematic representation for the 3′UTR of IGF-II transcript showing the exact position of binding to miR-615-5p. (b) Table for different binding sequences in the 3′UTR of IGF-II for miR-615-5p. Note that miR-615-5p seeding sequence is the same for all binding sites.

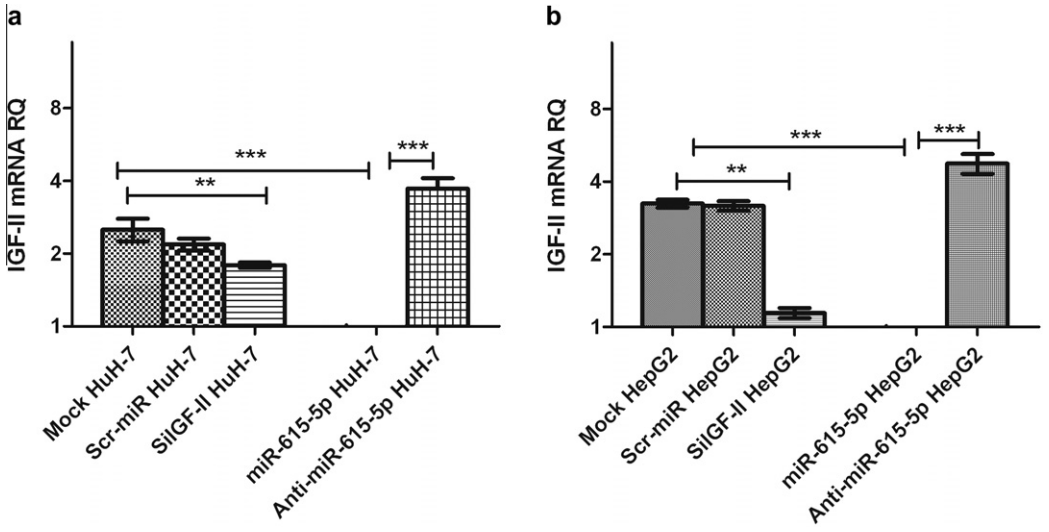


Fig. 6. IGF-II transcript as a common putative target of miR-615-5p; In HuH-7 and HepG2 cells: IGF-II mRNA expression was drastically suppressed upon mimicking of miR-615-5p compared to controls while inhibitors of miR-615-5p showed increased expression of IGF-II transcript levels compared to mimicked cells. IGF-II decreased upon using siRNAs against IGF-II compared to controls. One-way analysis of variance (ANOVA) was performed.

BrdU incorporation and MTT assays that stand in contrast to miR-155 which markedly induced proliferation and viability (Fig. 3). So miR-615-5p simulates both miR-122 and miR-199a/b-3p and they resulted in a potent HCC proliferation suppression [19,20]. miR-615-5p have also suppressed HCC migration in contrast to miR-155 which increased the migration (Fig. 4). So miR-615-5p acted like the tumor suppressor miR-338-3p and miR-198 in inhibiting HCC migration and invasion [21,22]. While miR-155 results were consistent with those previously obtained in promoting cellular migration and metastasis [8]. We further used anatagomirs for miR-615-5p to recover its inhibitory effect on the cell lines which showed increased proliferation and migration while antagonizing miR-155 showed repressed proliferative and migratory effect on the same cell lines.

Using in silico prediction for miR-615-5p downstream targets, we found that IGF-II as one of the potential targets for miR-615-5p. IGF-II is a pivotal mitogen in HCC that is frequently overexpressed in HCC and associates with hyperproliferation and tumor cell

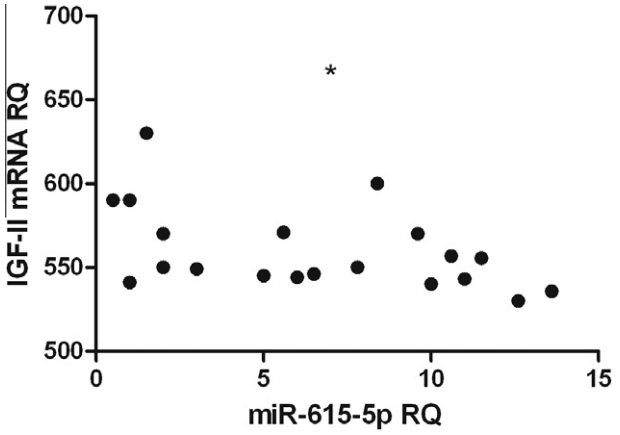


Fig. 7. Correlation analysis between miR-615-5p and IGF-II mRNA expressions in HCC tissues; Values of miR-615-5p and IGF-II mRNA RQ in HCC tissues were analyzed using Pearson method of correlation. An inverse correlation was found with Pearson $r = -0.468$ ($P = 0.0371$).

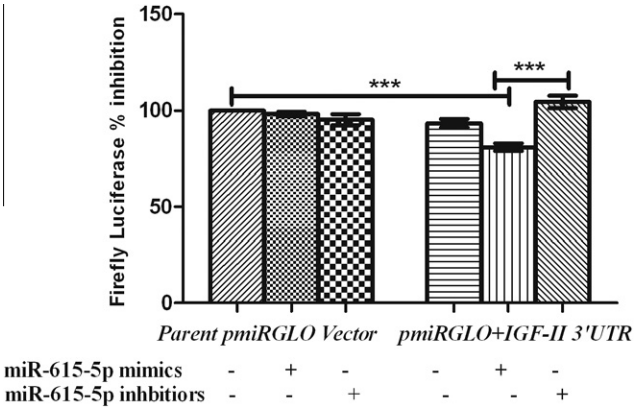


Fig. 8. IGF-II is a direct target of miR-615-5p; miR-615-5p mimics and inhibitors were co-transfected with pmiRGLO–insert or pmiRGLO + insert. Upon miR-615-5p mimics co-transfection, luciferase activity was inhibited by 19.4% in cells with the pmiRGLO + insert compared to cells transfected with the pmiRGLO–insert ($P < 0.0001$). Upon miR-615-5p inhibitors co-transfection, luciferase activity was increased by 9% compared to cells transfected pmiRGLO-insert ($P = 0.064$) and by 28% compared to pmiRGLO + insert co-transfected with miR-615-5p mimics ($P = 0.0007$).

migration [23,24]. Mathematical tools showed that miR-615-5p bind to 5 regions in the 3′UTR of the IGF-II mRNA by hitting with high binding scores by the same seed sequence (Fig. 5). Overexpressing miR-615-5p did not only reduce IGF-II transcript levels, but also phenocopied the effects of IGF-II knock down by siRNAs [23,25]. Using antagomirs for miR-615-5p, we were able to recover the inhibitory effect of miR-615-5p on IGF-II (Fig. 6). That was further supported by the shown inverse correlation between miR-615-5p and IGF-II in HCC tissues (Fig. 7). Although miR-615-5p was found to be expressed only in cancerous and cirrhotic tissues, but its expression was minimal in a way that cannot harness the overexpression of IGF-II (as an insufficient compensatory elevation). With the exception of a single descriptive study that examined the expression of miR-615 in prostate cancer [16], our results are the first to reveal the expression/function of miR-615-5p in HCC. We have shown that IGF-II is a direct target to miR-615-5p represented in the significant repression of luciferase activity upon miR-615-5p mimics co-transfection with the construct harboring the IGF-II 3′UTR target region of miR-615-5p (Fig. 8).

In conclusion, our data suggests that miR-615-5p might potentially serve as novel tumor suppressor microRNA in HCC. We show that miR-615-5p is restrictedly expressed in cirrhotic and HCC tissues (with higher abundance) but not in healthy liver tissues. miR-615-5p expression was inversely correlated with IGF-II mRNA expression in human HCC liver tissues. Furthermore, we have shown that IGF-II 3′UTR is a direct target for miR-615-5p that showed significant decrease in cell proliferation and migration upon its forced expression. Our results highly suggest miR-615-5p as promising tumor suppressor microRNA and might be a potential HCC diagnostic marker.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.06.054.

References

[1] Chen, M., Zhang, J., Li, N., Qian, Z., Zhu, M., Li, Q., Zheng, J., Wang, X. and Shi, G. (2011) Promoter hypermethylation mediated downregulation of FBP1 in human hepatocellular carcinoma and colon cancer. PLoS One 6, e25564.
[2] Feng, Y., Xue, W.J., Li, P., Sha, Z.Y., Huang, H., Rui, L., Li, H.X. and Mao, Q.S. (2011) RASSF1A Hypermethylation is associated with aflatioxin B1 and polycyclic aromatic hydrocarbon exposure in hepatocellular carcinoma. Hepatogastroenterology 59.
[3] Jia, Y., Yang, Y., Liu, S., Herman, J.G., Lu, F. and Guo, M. (2010) SOX17 antagonizes WNT/beta-catenin signaling pathway in hepatocellular carcinoma. Epigenetics 5, 743–749.
[4] Kong, L.M., Liao, C.G., Chen, L., Yang, H.S., Zhang, S.H., Zhang, Z., Bian, H.J., Xing, J.L. and Chen, Z.N. (2011) Promoter hypomethylation up-regulates CD147 expression through increasing Sp1 binding and associates with poor prognosis in human hepatocellular carcinoma. J. Cell Mol. Med. 15, 1415–1428.
[5] Ramzy, I.I., Omran, D.A., Hamad, O., Shaker, O. and Abboud, A. (2011) Evaluation of serum LINE-1 hypomethylation as a prognostic marker for hepatocellular carcinoma. Arab. J. Gastroenterol. 12, 139–142.
[6] Arbuthnot, P. and Thompson, L.J. (2008) Harnessing the RNA interference pathway to advance treatment and prevention of hepatocellular carcinoma. World J. Gastroenterol. 14, 1670–1681.
[7] Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
[8] Han, Z.B., Chen, H.Y., Fan, J.W., Wu, J.Y., Tang, H.M. and Peng, Z.H. (2012, Jan) Up-regulation of microRNA-155 promotes cancer cell invasion and predicts poor survival of hepatocellular carcinoma following liver transplantation. J. Cancer Res. Clin. Oncol. 138 (1), 153–161.
[9] Xie, Q., Chen, X., Lu, F., Zhang, T., Hao, M., Wang, Y., Zhao, J., McCrae, M.A. and Zhuang, H. (2012, May 1) Aberrant expression of microRNA 155 may accelerate cell proliferation by targeting sex-determining region Y box 6 in hepatocellular carcinoma. Cancer 118 (9), 2431–2442.
[10] Ying, Q., Liang, L., Guo, W., Zha, R., Tian, Q., Huang, S., Yao, J., Ding, J., Bao, M., Ge, C., et al. (2011) Hypoxia-inducible MicroRNA-210 augments the metastatic potential of tumor cells by targeting vacuole membrane protein 1 in hepatocellular carcinoma. Hepatology 54, 2064–2075.
[11] Park, J.K., Kogure, T., Nuovo, G.J., Jiang, J., He, L., Kim, J.H., Phelps, M.A., Papenfuss, T.L., Croce, C.M., Patel, T. and Schmittgen, T.D. (2011, Dec 15) MiR-221 silencing blocks hepatocellular carcinoma and promotes survival. Cancer Res. 71 (24), 7608–7616.
[12] Girard, M., Jacquemin, E., Munnich, A., Lyonnet, S. and Henrion-Caude, A. (2008) MiR-122, a paradigm for the role of microRNAs in the liver. J. Hepatol. 48, 648–656.
[13] Zhang S, Shan C, Kong G, Du Y, Ye L, Zhang X: MicroRNA-520e suppresses growth of hepatoma cells by targeting the NF-kappaB-inducing kinase (NIK). Oncogene 2011, Nov 21. doi: 10.1038/onc.2011.523.
[14] Wong, Q.W., Lung, R.W., Law, P.T., Lai, P.B., Chan, K.Y., To, K.F. and Wong, N. (2008) MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology 135, 257–269.
[15] He XX, Chang Y, Meng FY, Wang MY, Xie QH, Tang F, Li PY, Song YH, Lin JS: MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth in vitro and in vivo. Oncogene 2011, Nov 7. doi: 10.1038/onc.2011.500.
[16] Hulf, T., Sibbritt, T., Wiklund, E.D., Bert, S., Strbenac, D., Statham, A.L., Robinson, M.D. and Clark, S.J. (2011, Jan 21) Discovery pipeline for epigenetically deregulated miRNAs in cancer: integration of primary miRNA transcription. BMC Genomics 12, 54.
[17] Liu, S., Guo, W., Shi, J., Li, N., Yu, X., Xue, J., Fu, X., Chu, K., Lu, C., Zhao, J., et al. (2012, Feb) MicroRNA-135a contributes to the development of portal vein tumor thrombus by promoting metastasis in hepatocellular carcinoma. J. Hepatol. 56 (2), 389–396.
[18] Lin, J., Huang, S., Wu, S., Ding, J., Zhao, Y., Liang, L., Tian, Q., Zha, R., Zhan, R. and He, X. (2011, Nov) MicroRNA-423 promotes cell growth and regulates G1/S

transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma. Carcinogenesis 32 (11), 1641–1647.
[19] Zeng, C., Wang, R., Li, D., Lin, X.J., Wei, Q.K., Yuan, Y., Wang, Q., Chen, W. and Zhuang, S.M. (2010) A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. Hepatology 52, 1702–1712.
[20] Hou, J., Lin, L., Zhou, W., Wang, Z., Ding, G., Dong, Q., Qin, L., Wu, X., Zheng, Y., Yang, Y., et al. (2011) Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell 19, 232–243.
[21] Huang, X.H., Chen, J.S., Wang, Q., Chen, X.L., Wen, L., Chen, L.Z., Bi, J., Zhang, L.J., Su, Q. and Zeng, W.T. (2011, Nov) MiR-338-3p suppresses invasion of liver cancer cell by targeting smoothened. J. Pathol. 225 (3), 463–472.
[22] Tan, S., Li, R., Ding, K., Lobie, P.E. and Zhu, T. (2011) MiR-198 inhibits migration and invasion of hepatocellular carcinoma cells by targeting the HGF/c-MET pathway. FEBS Lett. 585, 2229–2234.
[23] Nussbaum, T., Samarin, J., Ehemann, V., Bissinger, M., Ryschich, E., Khamidjanov, A., Yu, X., Gretz, N., Schirmacher, P. and Breuhahn, K. (2008) Autocrine insulin-like growth factor-II stimulation of tumor cell migration is a progression step in human hepatocarcinogenesis. Hepatology 48, 146–156.
[24] Breuhahn, K. (2010) Molecular mechanisms of progression in human hepatocarcinogenesis. Pathologe 31 (Suppl 2), 170–176.
[25] Lund, P., Schubert, D., Niketeghad, F. and Schirmacher, P. (2004) Autocrine inhibition of chemotherapy response in human liver tumor cells by insulin-like growth factor-II. Cancer Lett. 206, 85–96.