EXPLORING ROLES OF MIR-372-3P IN PROLIFERATION OF HEPATOCELLULAR CARCINOMA CELLS

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, and it has been considered as the second leading cause of cancer-related death. Many factors were shown to be associated with HCC development such as hepatitis virus infection, alcohol consumption, and nonalcoholic steatohepatitis (NASH). As well as the other types of cancer, HCC also shares a typical cancer hallmark including indefinite proliferation, avoiding growth suppressers, and activation of replicative immortality. In addition, cell cycle-related protein regulating proliferation in cancer are often found dysregulated, allowing cancer cells to proceed their proliferation uncontrollably. Recently, a small non-coding RNA, microRNA (miRNA), was found to play an important role in numerous biological functions. A particular miRNA may ameliorate or promote cancer progression through different target mRNA, suppressing translation into protein. MiR-372-3p has been widely explored in various cancers such as colon cancer, colorectal cancer, and glioma. However, its functions have been rarely explored in HCC, especially in the aspect of cancer proliferation. This study, thus, aims to investigate its role of in HCC cell line proliferation by introducing vector containing miR-372-3p sequence to these cell lines and evaluating their proliferation activity, respectively. The preliminary results indicated that normal hepatocyte cell line exhibited lower expression of miR-372-3p compared to HCC cell lines. Moreover, decelerated proliferation rate was found in transduced HCC cell lines compared to control.

Keywords: MicroRNA-372-3p, Hepatocellular Carcinoma, Cell Cycle, Cell Proliferation

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common liver malignancies, is currently leading to cancer-related death occuring worldwide. It was also reported as the ninth cause of cancer deaths in USA and as the fourth globally (CDC, 2010; Yang et al., 2019). Hepatitis virus infection, nonalcoholic steatohepatitis (NASH), obesity, liver fibrosis, and chronic liver diseases are the essential risk factors leading to HCC (Balogh et al., 2016; Llovet et al., 2021). Several internal factors have been discovered to positively influence HCC development, such as changes in cellular microenvironment, interactions between non-viral and viral risk factors, genetic mutations, modulation of immune cells, and severity of the ongoing chronic liver disease (Llovet et al., 2021).

Developed by Weinberg, cancer hallmarks represent many unique characteristics including sustaining proliferative signaling, evading growth suppressors, and enabling replicative immortality. Unlike normal cell that tightly regulated by growth-signals to maintain the balance in cell number, cancer cell becomes independent from those growth-signals (Hanahan & Weinberg, 2011). To facilitate limitless cell growth, cell cycle-related proteins are found to be overexpressed in cancer cells such as cyclin D, -E, and cyclin-dependent kinase (CDK) 2, -4, -6. Nevertheless, previous studies showed that tumorigenesis can be impaired by carcinogenic treatment or genetic manipulation. By targeting CDK 4 and CDK6, which is required to maintain cancer homeostasis, tumorigenesis could be impaired (Fry et al., 2004).

Recently, a non-coding RNA with 18-24 nucleotides length, micro-RNA (miRNA), was discovered to serve an important role in several biological activities including cell growth, cell proliferation, or apoptosis (Szabo & Bala, 2013). Mechanistically, miRNA binds to mRNA target to either modulate upregulation or downregulation of the target (Dharap et al., 2013; Huntzinger & Izaurralde, 2011). Several miRNAs have been identified to be strongly involved with HCC pathogenesis, some of which are carcinogenic miRNAs or anti-carcinogenic miRNAs. For example, miR-181c-5p, miR-151, and miR-221-3p are classified as carcinogenic miRNAs (Abd ElAziz et al., 2022; Ding et al., 2010; Tan et al., 2022), whereas miR-199, miR-29, and miR-26b-5p are included in anti-carcinogenic miRNAs (Wang et al., 2016; Zhang et al., 2017; Zhang et al., 2018). Interestingly, numerous number of miRNAs has been known to modulate cancer progression through cell cycle-related proteins. To illustrate, miR-125b targets CDC25A, and E2F3 to initiate cell cycle arrest at G1/S transition. However, downregulation of miR-125b is often found in many types of cancer including glioma stem cells, and bladder cancer (Huang et al., 2011; Shi et al., 2010). In pituitary adenoma, miR-128a, miR-155, and miR-516a-3p were found to suppress Wee1 kinase which is required to delay mitosis. These miRNAs are thus considered as anti-carcinogenic miRNAs (Butz et al., 2010).

Liver fibrosis and its later stage, liver cirrhosis, can lead to the development of HCC (Friedman, 2003). Residing within the space of Disse, hepatic stellate cells (HSCs) remain in quiescent state and feature several retinoid lipid droplets. Hepatocyte injury resulting from toxic or viral infection triggers the alteration of HSC phenotype into collagen-producing myofibroblasts or activated HSC. In response to hepatocyte injury, myofibroblasts migrate to the site of injury and secrete extracellular matrix (ECM).

MiR-372-3p has been widely investigated in many cancers such as prostate cancer, colon cancer, colorectal cancer, and HCC (Li et al., 2022; Peng et al., 2019; Wu et al., 2014; Yin et al., 2022). However, its role in HCC proliferation has not been fully elucidated, especially in the aspect of cell cycle regulation. This study aims to discover the underlying function of miR-372-3p in modulating cancer progression through cell-cycle related protein. By using web-based bioinformatic tools such as TargetScan, miRTarBase, MirSystem, and DIANA, the possible mRNA targets could be predicted.

LITERATURE REVIEWS

Incidences and Causes of Hepatocellular carcinoma (HCC)

HCC is the fourth common leading cause of cancer-related death worldwide, especially in the country where medical resources and quality of lifecare have been limited, such as, sub-Saharan Africa and Eastern Asia (Tang et al., 2018; Yang et al., 2019). From 2005 to 2015, HCC was the second underlying cause of death, after lung cancer (Tang et al., 2018; Yang et al., 2019).

Infection of hepatitis B and C virus (HBV, HCV) is the major contribution to HCC pathogenesis, up to 80% of HCC patients worldwide (El-Serag, 2012; Yang & Roberts, 2010). Several studies have demonstrated that individual harboring hepatitis B virus has 10-25% lifetime risk to develop HCC (Crissien & Frenette, 2014).

Another contributor that leads to HCC is nonalcoholic fatty liver disease (NAFLD). NAFLD contains a broad spectrum of many liver diseases including nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver (NAFL) and liver fibrosis and cirrhosis (Burt et al., 2015). NAFL features steatosis, an excessive triglyceride accumulation in hepatocytes, and could develop into NASH, a following phase that exhibits hepatocyte injury and mild inflammation. In addition, both NAFL and NASH have a potential to influence the formation of liver fibrosis and cirrhosis and ultimately HCC (Anstee et al., 2019).

Cell Cycle and Related Proteins

Cell cycle is divided into four principal phases such as G1, S, G2, and M phase. G1, S, and G2 phase are included in sub-phase interphase, whereas M phase can be further divided into mitosis and cytokinesis. There are five states in mitosis: prophase, prometaphase, metaphase, anaphase, and telophase (Panagopoulos & Altmeyer, 2021; Wang, 2021). The transition to the next phase and the progression of the cell cycle is primarily regulated by cyclin dependent kinase (CDK) and cyclin. CDK, a kinase controlled by the phosphorylation at tyrosine and threonine residues, forms a complex with cyclin to either becomes activated or inactivated, depending on the type of cyclin they associated (Schafer, 1998).

During the G1 phase, CDK4 or CDK6 associate with cyclin D, establishing a cyclin D-CDK4/6 complex, to activate the complex and further partially phosphorylates downstream substrate, retinoblastoma protein (RB) (Lundberg & Weinberg, 1998; Sherr & Roberts, 1999). The phosphorylation of RB leads to the detachment of its target, E2F, which is a critical transcription factor in facilitating early cell cycle genes (Arroyo & Raychaudhuri, 1992). Detached E2F upregulates the expression of cyclin A and cyclin E. CDK2 joins cyclin E to form a cyclin E-CDK2 complex and then fully phosphorylates RB, respectively, allowing cell to proceed to the S phase (Lundberg & Weinberg, 1998). In the S phase, cyclin A binds to CDK2 to establish a cyclin A-CDK2 complex which is required to stimulate the proteins responsible for DNA synthesis (Petersen et al., 1999). After entering the G2 phase, cyclin B-CDK1 complex gradually increases from the beginning to the end of the phase. The rising of cyclin B-CDK1 complex, maintained by cyclin A-CDK1 complex, drives cell cycle to the M phase. Cyclin B-CDK1 complex starts to decline continuously throughout the M phase, promoting the completion of mitosis (Petersen et al., 1999).

MicroRNAs (miRNAs) and Its role in HCC

Following studies revealed that miRNA is non-coding RNA with 18-24 nucleotides in length and it acts as post-transcriptional regulator, controlling gene expression. The biogenesis of miRNA usually initiates with transcription by RNA polymerase II, yielding primary miRNA (pri-miRNA), before entering hairpin-like structure formation via canonical pathway using Drosha protein. This hairpin-like structure, precursor miRNA (pre-miRNA), is then exported to the cytoplasm and further processed by Dicer to cleave hairpin loop, resulting in imperfect miRNA duplexes. Cleaved miRNA duplexes interact with Argonaute family proteins including

Ago 1-4 to establish a miRNA-induced silencing complex (miRISC), in which strand selection takes place (Ha & Kim, 2014; Wang et al., 2012).

Several biological processes, such as cell metabolism, cell proliferation, cell apoptosis, cell necrosis, and epithelial-mesenchymal transition (EMT) in cancer, have been known to modulated by miRNAs (Szabo & Bala, 2013). Previous studies have indicated several cell cycle protein targets of miRNA in HCC. Overexpression of miR-124 or miR-203 in HCC cell lines revealed many downregulated target proteins including CDK6, suggesting its role as an anti-cancer miRNA (Furuta et al., 2010). By targeting 3'-UTR of p21Cip1/Waf1, oncogenic miR-423 drives cell cycle progression from the G1 to S phase and stimulates HCC growth (Lin et al., 2011). As found upregulated in HCC, miR-221 binds to 3'-UTR CDKN1B/p27 and CDKN1C/p57, a cyclin-dependent kinase inhibitor (CDKI), to increase the number of cell in the S phase and promote cell proliferation (Fornari et al., 2008).

MiRNA-372-3p and Its Function in Various Cancers

Transcribed from the MiR-371-372 gene cluster on chromosome 19q13.42, miR-372 has been known to be involved with many cancer cell activities such as cell proliferation, cell migration, cell invasion, and apoptosis. MiR-372 may serve as either oncogenic miRNA or anti-oncogenic miRNA in a particular type of cancer (Zhao et al., 2017). In addition, miR-372 has also been shown to correlate with poor prognosis in glioma and HCC (Li et al., 2013; Wu et al., 2015). Upregulating miR-372 indicated its role as oncogenic miRNA and may regulate aggressive development in gliomas (Li et al., 2013). Previous studies have explored the mechanistic function and target mRNA of miR-372 in various cancers. To demonstrate, In colon cancer cell line, miR-372-3p binds to 3'-UTR of MAP3K2, preventing the mediation of MAPK signaling and thus inhibiting cell proliferation (Li et al., 2022). Inducing the expression of miR-372-3p in osteoblastic cell line could prohibit cell proliferation and cell metastasis via regulating FXYD domain-containing ion transport regulator 6 (FXYD6) (Xu et al., 2018). Suppression of miR-372-3p mediates Hippo signaling through LATS2 which inhibits colorectal cancer proliferation, migration, and invasion (Peng et al., 2019). From the literature review, the conceptual framework can be drawn as shown in Figure 1.

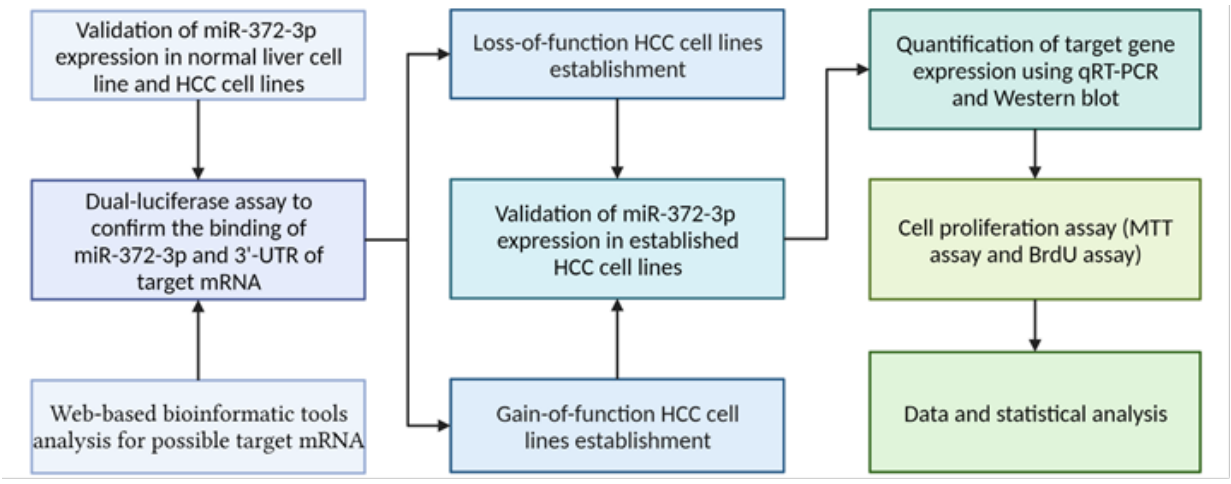


Figure 1 Conceptual framework

RESEARCH METHODOLOGY

Cell Culture

HepG2 was cultured in low-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin). JHH4 cell line was cultured in Eagle's minimum essential media (EMEM) with the same supplements as in HepG2 culture. All cells were maintained at 37°C with 5% CO_2.

Firefly-Renilla Luciferase Assay

pmirGLO Dual-Luciferase was used to clone wild-type and mutant 3'-UTR of CCND1, and pSilencer was used to express miRNA-372-3p expression. HEK293FT cell was seeded into 96-well plate at $1x10^4$ cells in concentration before transfection of the cloned plasmids. Empty pmirGLO and empty pSilencer were co-transfected and used as control. Cloned wild-type 3'-UTR pmirGLO and cloned pSilencer were co-transfected and used as wild-type. Cloned mutant 3'-UTR pmirGLO and cloned pSilencer were co-transfected and used as mutant. After co-transfection, the culture was subjected to luciferase assay using Dual-Luciferase® Reporter Assay kit (Promega). The experiment was carried out according to manufacturer protocol.

Cell Proliferation Assay

MTT Assay

Cells were seeded into 96-well plate in concentration of $5x10^3$ cells per well. MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide)(Sigma-Aldrich) was prepared in final concentration of 0.5 mg/ml in low glucose DMEM without FBS and streptomycin and penicillin. Cells were incubated at 37°C with 5% CO_2 for 30 minutes. Media was removed and replaced by 100 µl dimethyl sulfoxide (DMSO)(Sigma-Aldrich), respectively. The culture was then incubated at 37°C with 5% CO_2 for 5 minutes without exposure of light. The absorbance of each well was analyzed by BioTek™ Synergy™ HTX Milti-mode Microplate Reader (Thermo Fisher Scientific) using wavelength of 570 nm.

BrdU Assay

Cells were seeded into 24-well plate in concentration of 1 x 10^5 cells/ml and were incubated for 48 hours. Cell medium was replaced by 500 µl 10 µM BrdU labeling solution, and cells were incubated at 37°C with 5% CO_2 for a desired time. The labeling solution was removed. After washing with PBS, 500 µl 4% formaldehyde in PBS was added to the culture before the addition of 500 µl Triton X-100 permeabilization buffer. The culture was replaced with 500 µl 2N HCl and 500 µl blocking buffer was added to the culture, respectively. Blocking buffer was removed and 500 µl of blocking buffer with anti-BrdU primary antibody (sc-32323; Santa Cruz) in 1:200 ratio was added to the culture. Secondary antibody (1:500 dilution) and DAPI (1:1000 dilution) were added. Washing with Triton X-100 permeabilization buffer was then proceeded before the addition of PBS to the culture. The cells were observed under the fluorescence microscope at 10x magnification. The number of BrdU stained cells was normalized by the number of total DAPI, per field.

Statistical analysis

Data are represented as mean $\pm$ standard deviation (SD), and the difference between experimental group and control group was compared using Student's t-test from GraphPad Prism version 5 (San Diego, CA, USA). The differences with $*p\ value < 0.05$, $**p\ value < 0.01$, and $***p\ value < 0.001$ were considered significant.

RESEARCH RESULTS

Web-based Bioinformatic Tools Revealed the Possible Interaction between MiR-372-3p and Cell Cycle-related Target MRNAs.

Four web-based bioinformatic tools were utilized to retrieve the possible interactions between miR-372-3p and cell cycle-related target mRNAs. TargetScan predicted the interaction of miR-372-3p with CDK2, CCND1, CCND2, E2F1, E2F2, E2F3, E2F5, and CDC25A while miRTarBase only discovered the interaction with CDK2, and CCND2. CCND1, CCND2, E2F1, E2F3, and E2F5 were predicted using MirSystem. DIANA also predicted CCND1, E2F1, E2F2, and E2F5.

Table 1 Web-based bioinformatic programs, TargetScan, MirTarBase, MirSystem, and DIANA, which are used to predict the interaction of miRNA-372-3p and its cell cycle-related mRNAs.

	TargetScan	**MirTarBase**	**MirSystem**	**DIANA**
Cyclin-dependent kinase 2 (*CDK2*)	+	+	-	-
Cyclin D1 (*CCND1*)	+	-	+	+
Cyclin D2 (*CCND2*)	+	+	+	-
E2 factor 1 (*E2F1*)	+	-	+	+
E2 factor 2 (*E2F2*)	+	-	-	+
E2 factor 3 (*E2F3*)	+	-	+	-
E2 factor 5 (*E2F5*)	+	-	+	+
Cell division cycle 25 homolog A (*CDC25A*)	+	-	-	-

Decreased MiR-372-3p Expression Was Found in HCC Cell Line.

QPCR analysis was used to investigate the nature of miR-372-3p expression in different HCC cell lines and normal hepatocyte cell line. THLE2, a normal hepatocyte cell line, showed a significant higher expression of miR-372-3p compared to HepG2 and JHH4 which are characterized as HCC cell line (Figure 2).

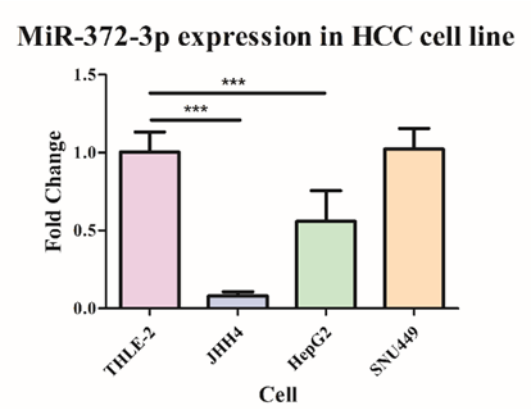


Figure 2 MiR-372-3p expression in THLE-2, JHH4, and HepG2 cell line. Data are shown as mean + SD. ***$p < 0.001$.

Transduced HCC Cell Line Expressed a Higher Level of MiR-372-3p.
To investigate the expression of miRNA-372-3p in established HCC cell lines, all cells were subjected to qPCR analysis. Transduced HepG2 exhibited a higher expression of miRNA-372-3p compared to wild-type. As well as in transduced JHH4 and SNU449 cell line, both of them showed a significantly higher expression of miRNA-372-3p than their wild-type counterpart (Figure 3).

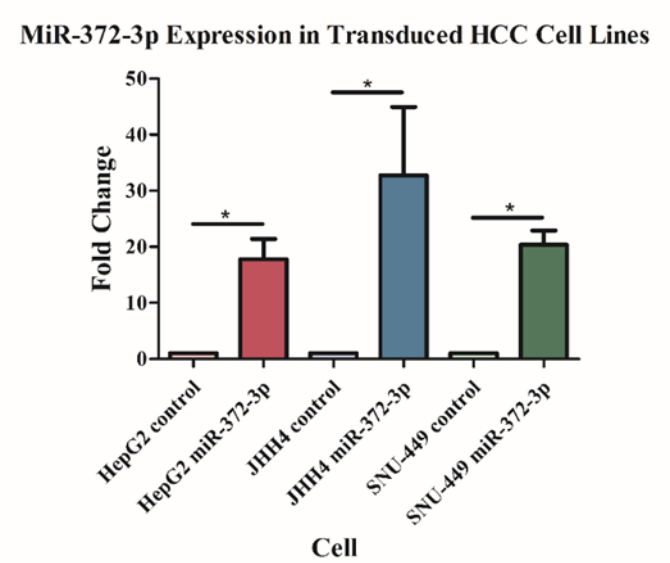
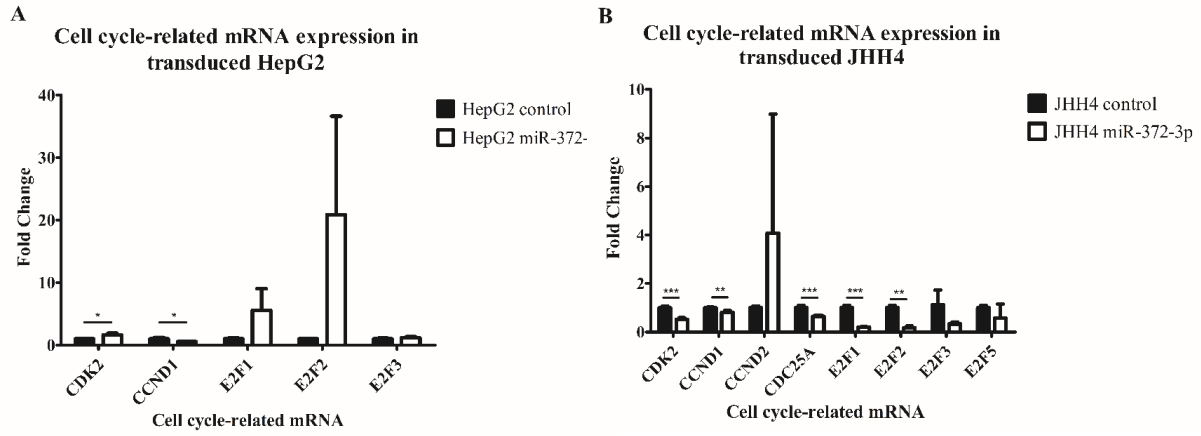


Figure 1 The expression of miR-372-3p in different transduced HCC cell line. Data are represented as mean ± SD. *$p < 0.05$.

Changes in Cell Cycle-Related mRNA Were Detected in Established HCC Cell Line.
MRNA expression of major proteins involved with cell cycle was proceeded in different established HCC cell lines using qPCR quantification. Validated major proteins regulating cell cycle progression included CDK2, CCND1, E2F1, E2F2, and E2F3. In JHH4, these proteins were majorly downregulated compared to control. Transduced HepG2, however, showed different results. Only CCND1 expression was downregulated whereas CDK2, E2F1, and E2F2 possessed a higher expression and E2F3 mRNA level remained unchanged compared to control. Interestingly, transduced SNU-449 exhibited several upregulated cell cycle-related mRNA expressions such as CDK2, CCND1, CDC25A, E2F1, E2F2, and E2F3 (Figure 4).



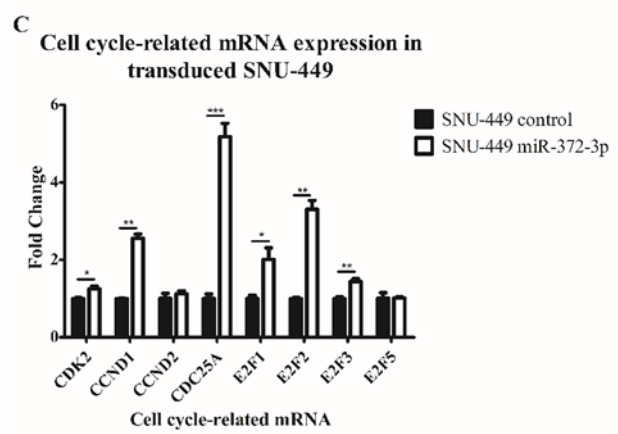


Figure 4 (A) Expression of cell cycle-related mRNA in transduced JHH4. (B) Expression of cell cycle-related mRNA in transduced HepG2. (C) Expression of cell cycle-related mRNA in transduced SNU-449. Data are showed as mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

MiRNA-372-3p Decreased CCND1 MRNA Expression.

According to previous qPCR data, CCND1 mRNA expression was the only mRNA that was downregulated in all transduced HCC cell lines. Luciferase assay was then used to determine the interaction between miR-372-3p and CCND1 mRNA. The results indicated that luciferase activity is the weakest in wild-type group while mutant group showed a significantly higher luciferase activity. Moreover, luciferase activity of wild-type group was also significantly lower than that of mutant group (Figure 5).

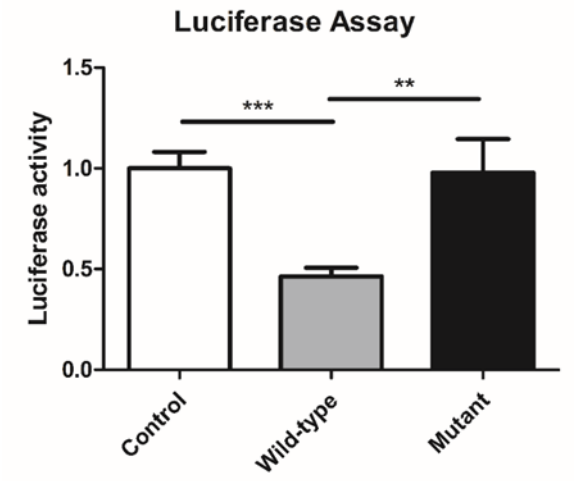


Figure 5 Luciferase activity. Data are represented as mean + SD. **$p < 0.01$; ***$p < 0.001$.

Deterred HCC Cell Line Proliferation as a Result of MiRNA-372-3p Overexpression

MTT assay was carried out to determine the change in proliferation rate of transduced HCC cell lines. The proliferation rate of transduced HepG2 was significantly decreased compared to that of control, especially in day 2. Transduced JHH4 began to decline their proliferation rate at day 2, and day 3. In addition, transduced SNU-449 showed a lower proliferation rate since day 1 (Figure 6).

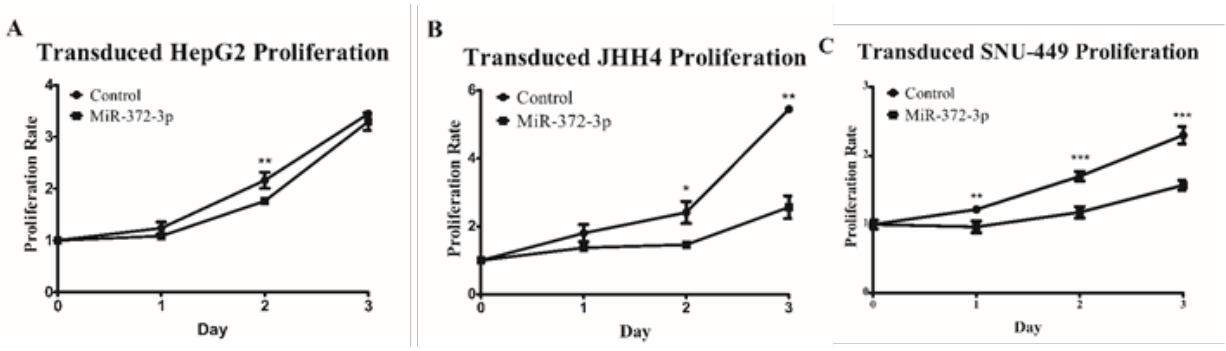


Figure 6 MTT assay of transduced (A) HepG2, (B) JHH4, and (C) SNU-449. Data are represented as mean + SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

To narrow down the phase of cell cycle affected by overexpression of miR-372-3p, BrdU assay was then performed. Interestingly, the transduced SNU449 cell line showed a lower BrdU/DAPI ratio compared to that of control (Figure 7).

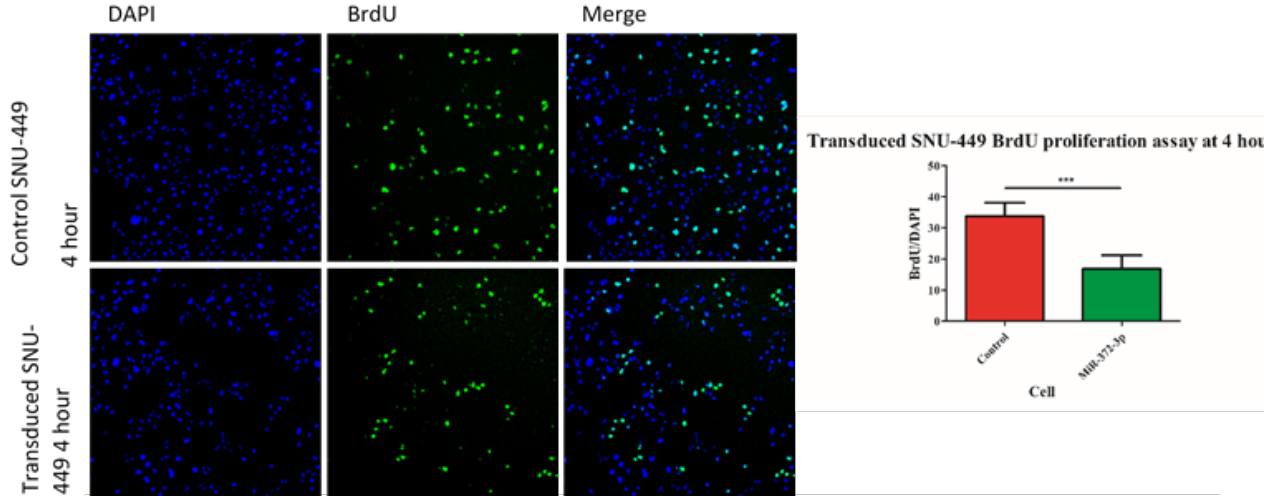


Figure 7 BrdU assay of transduced SNU-449 at 4 hour. Data are showed as mean + SD. ***$p < 0.001$

DISCUSSION & CONCLUSION

The roles of miR-372-3p seem to vary in each type of cancer. As depicted in previous research, miR-372-3p may have an anti-oncogenic property in colon cancer cell line but it turns into oncogenic roles in lung squamous cell carcinoma. However, their functions in HCC have been rarely explored.

The prediction from web-based bioinformatic programs, TargetScan, miRTarBase, miRSystem, and DIANA, indicates that miR-372-3p may hold a potential to interfere with the following cell cycle-related mRNAs; CCND1, CCND2, CDC25A, CDK2, E2F1, E2F2, and E2F3. Thus, investigating these mRNA expressions in HCC cell lines which are transduced to overexpress miR-372-3p is essential. After introducing the vector carrying miR-372-3p premature sequence to HCC cell lines, qPCR analysis showed that all the transduced cell lines produced miR-372-3p in a higher level compared to control which was introduced with vector containing mCherry sequences, a red fluorescence protein. Thus, the production of miR-372-3p in established cell lines was confirmed.

Validation of cell-cycle related mRNA expression in established HCC cell line was then proceeded, investigating the effects of miR-372-3p overexpression. In transduced JHH4, predicted mRNAs were majorly downregulated except for CCND2. Combined with the results

retrieved from MTT assay, it could be implied that miR-372-3p might interfere with JHH4 cell line proliferation through the interaction with one or more of their mRNA targets. As well as in JHH4, HepG2 proliferation rate also tends to be significantly declined at day 2. However, qPCR results were different. Only CCND1 mRNA that was downregulated by miR-372-3p overexpression. Luciferase assay was then employed to investigate the effect of miR-372-3p in CCND1 mRNA expression. Decreased luciferase activity in wild-type group compared to that of mutant group suggested that miR-372-3p could target 3'-UTR of CCND1 mRNA. Despite the several upregulated cell cycle-related mRNA expression in transduced SNU-449, MTT assay indicated a significant reduction in proliferation rate. Moreover, BrdU assay also revealed that the overexpression of miR-372-3p might impede the progression of transduced SNU-449 cell cycle to the S-phase.

For future experiments, transduced HepG2 and transduced JHH4 were planned for BrdU assay. The additional cell-cycle related mRNAs were also planned for transduced HepG2. Once the interaction of miR-372-3p and its target has become clear, western blot of the target protein could reveal the post-translational effect of miR-372-3p overexpression in each transduced HCC cell line. Loss-of-function could also confirm the role of miR-372-3p by suppressing the expression of miR-372-3p in HCC cell line and evaluating the proliferation rate, respectively.

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