

WNT1 Gene from WNT Signaling Pathway Is a Direct Target of miR-122 in Hepatocellular Carcinoma

Zeinab Ahsani¹ · Samira Mohammadi-Yeganeh^{2,3} ·
Vahid Kia⁴ · Hamzeh Karimkhanloo¹ ·
Nosratollah Zarghami^{1,5} · Mahdi Paryan⁶

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Abstract Hepatocellular carcinoma (HCC) is an invasive form of hepatic cancer arising from the accumulation of multiple genetic alterations. In this study, the causal role of disturbed canonical Wnt/ β -catenin pathway was approved, and some of HCC-driven important gene candidates were determined. MicroRNAs (miRNAs), small non-coding RNAs, are the key regulators of important cancer genes, and their participation in tumorigenesis has been shown. By reviewing literature, *WNT1* gene with functional significance was selected to approve miRNAs as new subjects for targeted therapy.

For proper and fast miRNA detection and also confirmation of the role of bioinformatics in obtaining practical data, we benefited from different bioinformatics tools such as TargetScan, miRanda, and DIANA. In order to use an HCC model, we used HepG2 cell line. Luciferase assay was applied to assess the ability of the selected miRNAs in targeting *WNT1* 3'-UTR. To

✉ Nosratollah Zarghami
Zarghami@tbzmed.ac.ir

✉ Mahdi Paryan
m_paryan@pasteur.ac.ir

¹ Department of Medical Biotechnology, Faculty of Advanced Medical sciences, Tabriz University of Medical Sciences, Tabriz, Iran

² Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

³ Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁴ Department of Medical Biotechnology and Nanotechnology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

⁵ Department of Clinical Biochemistry and Laboratory Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

⁶ Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, Tehran, Iran

overexpress the selected miRNA in HepG2 cell line, viral construct was prepared. Quantitative real-time PCR was performed to evaluate selected miRNA and target gene expression levels. miR-122 was selected according to data concerning various bioinformatics tools.

miR-122 was downregulated and *WNT1* gene expression was upregulated in HepG2 cell line. After viral construct transduction, miR-122 expression was elevated and *WNT1* expression was notably declined. Finally, we introduced *WNT1* gene as one of the important genes in HCC, and also, we showed that miR-122 can regulate *WNT1* gene expression.

Moreover, our study determines the potential of bioinformatics analyses in providing accurate and reliable data for miRNA: messenger RNA (mRNA) prediction.

Keywords Hepatocellular carcinoma · Wnt signaling · Bioinformatics · WNT1 · miR-122

Introduction

MicroRNAs (miRNAs) are a group of small non-coding, single-stranded RNAs, which are 19–25 nucleotides long [1, 2]. Their major role is to degrade or suppress messenger RNAs (mRNAs) through perfect or partial binding to mRNA's 3'-UTR, respectively. In mammals, miRNAs are responsible for the silencing of nearly 50 % of protein-coding genes post-transcriptionally [3–5]. So far, hundreds of miRNAs have been discovered in plants and vertebrates using molecular cloning and bioinformatics approaches [6, 7]. Since 2002, the scores of research have revealed that point mutations, deletions, and insertions in miRNA genes play a part in the etiology of cancers [8]. miRNAs have a significant role in fundamental biological processes, such as cell growth, division, and differentiation as well as apoptosis and metastasis [9–11]. Based on microarray data, they can be used as potential biomarkers in different types of cancers [12]. In addition, miRNAs have been considered markers for tumor classification and grading [13]. Furthermore, they have been regarded as oncogenes, tumor suppressor genes, and stem cell modulators [14].

miRNAs have an extensive role in primary hepatic cancer, or hepatocellular carcinoma (HCC) [6], which is the seventh among malignancies and is the second cause of cancer-related death. The incidence of HCC is 750,000, and 700,000 individuals die of HCC annually [4]. HCC is refractory, recurrent, and metastatic. In addition, its mortality rate is high, and it cannot be diagnosed until late stages [15]. Overall, prognosis and diagnosis of HCC is possible only in late stages when treatments have the lowest efficacy. Thus, there is an urgent need for markers that can help in diagnosing HCC in primary stages [16]. miRNAs target and regulate the expression of mRNAs of proteins that are involved in cell cycle, apoptosis, and HCC metastasis. Given the microarray results, some of miRNAs have been up- or downregulated. Their downregulation, however, is more common in HCC. This, in fact, shows that some of miRNAs act as tumor suppressor genes, and their re-establishment can prevent cell cycle and enhance apoptosis. In addition, by inhibiting cell migration and invasion, they prevent angiogenesis and metastasis [17]. In HCC, some of the signaling pathways that control cell division, angiogenesis, invasion, and metastasis are dysregulated [18]. Research indicates that most of the mutations that continually activate Wnt/ β -catenin are related to human tumors, which is indicative of the pathway's role in cancer development [19]. WNT/ β -catenin signaling pathway has a pivotal role in growth, development, and differentiation of HCC and is one of the most prominent ones that is frequently afflicted in cancers. Clinical investigations indicate the aberrant activation of WNT/ β -catenin in 40–70 % of HCC cases. Therefore, it is regarded as a major therapeutic target in various clinical studies [20–23]. Upon

the binding of ligands to Wnt pathway receptors, i.e., Frizzled (FZD) receptors, a cascade of signals is initiated resulting in the accumulation of β -catenin in cytoplasm and subsequently entering the nucleus [24]. In the nucleus, it interacts with other transcription factors, which leads to the activation of target genes including *c-MYC* oncogene [25]. If any of the proteins involved in the pathway is corrupted, β -catenin is abnormally accumulated in the nucleus, and the targets are upregulated which leads to tumorigenesis [26]. Previous studies show a relationship between anomalous expression of *WNT* gene family and human tumorigenesis. *WNT1* is a member of *WNT* family that intensifies cancer progression by promoting cell cycle, migration, and survival [25]. For example, when *WNT1* suppressor genes are hyper methylated or deleted, expression of *WNT1* increases, a phenomenon observed in various cancers [27]. Considering the substantial role of *WNT1* in different types of cancers including HCC, we selected it as the target of miRNAs in the present study.

There are various methods, such as Northern blot, microarray, real-time PCR, and in situ hybridization to quantify miRNAs in tissues and to explore their role in malignancies [28]. However, since not capable of differentiating between pri-, pre-, and mature miRNAs and their families, these methods are not used extensively. In addition, although researchers can obtain a complete expression profile using microarray, it cannot be used conveniently in developing countries because of its high cost [29].

Microarray is a high throughput and reproducible technology. The applications of this technology facilitate simultaneously determining the expression levels of large numbers of targets in a single experiment. However, its specificity and sensitivity are limiting factors in miRNA expression profiling [2, 30].

Bioinformatics algorithms and in silico modeling systems have also been applied to predict miRNA profiling and can provide useful information on the role of miRNAs in molecular pathways involved in cancer [7]. There are a variety of software applications that predict mRNA-targeting miRNAs [31, 32]. The sequence based approaches predict large numbers of miRNAs for different targets based on imperfect complimentary between miRNA and 3'-UTR of mRNA of interest. In addition, since there is a lot of miRNAs that are dysregulated in different types of cancers, it will be more rapid and cost-effective to use bioinformatics approaches for the prediction of miRNAs targeting mRNAs of cancer-related genes [11]. This approach is applied by many web-based target prediction software applications, such as TargetScan, PicTar, miRanda, and DIANA-microT. Although the data retrieved by microarray and computational approaches are useful, they should be finally confirmed by more specific alternative methods [29]. In the present study, we exploited various bioinformatics algorithms to predict miRNAs that target *WNT1* mRNA and confirmed the bioinformatics predictions experimentally. Furthermore, to address the mentioned downstream validation challenge, we used the approved home-brew stem-loop RT primers as novel cost-effective RT-PCR assay for detection of miRNA expression profile.

Materials and Methods

miRNA Prediction

The sequence of *WNT1* gene was retrieved from NCBI (www.ncbi.nlm.nih.gov), and then, the most popular miRNA bioinformatics software applications, such as TargetScan (<http://www.targetscan.org>), PicTar (<http://pictar.mdc-berlin.de>), Miranda (<http://www.microrna.org>), and

Microcosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5>) were used to predict miRNAs targeting *WNT1* gene. We also used DIANA lab software, which has several programs with specific algorithms and considerations for miRNA selection. Then, for data validation, we utilized miRWalk software (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk>), which is a comprehensive atlas of predicted and validated miRNA-mRNA interactions. In addition, we recruited Qiagen database (<http://sabiosciences.com>) which provides a list of miRNAs involved in different cancers. We searched through all these software applications with different approaches about miRNA prediction. Afterwards, we made a score table to select the best candidate miRNAs for subsequent experimental analyses.

Cell Lines

HepG2 (human hepatocellular carcinoma cell line) and HEK293T cell lines were purchased from the National cell bank of Iran (Pasteur Institute of Iran, Tehran, Iran) and cultured in DMEM culture medium supplemented with 5 and 10 % fetal bovine serum (FBS), respectively, in 37 °C with humidified atmosphere and 5 % CO₂.

All the procedure of the study has been approved by Ethics Committee of the Pasteur Institute of Iran, Tehran, Iran and has been confirmed by the provisions of the Declaration of Helsinki.

Lentiviral Packaging and Cell Transduction

HEK293T cell line, as host cells for high titer virus production, was cultured in DMEM containing 10 % FBS. Cell transfection was performed according to calcium phosphate method developed by Trono lab protocols [33] using pMD2G (encoding the VSV G envelope protein), psPAX (packaging vector), and miR-122-containing vector or an empty control vector pLenti-III. miR-122-containing vector was purchased from Stem Cell Technology Research Center, Tehran, Iran. Assembled viruses stacked in supernatant were harvested every 24 h for 3 days and stored at 4 °C for later transduction. Viruses were filtered and concentrated using ultra-centrifugation at 40,000 *g* (23,000 rpm) for 2 h and 30 min at 4 °C. Polybrene (4 µg/ml) was added to increase transduction efficiency. HepG2 cells were cultured in DMEM +5 % FBS and transduced by lentiviruses. Culture medium was changed with fresh medium after 6–8 h because of polybrene toxicity for the cells. Antibiotic treatment for the selection of transduced cells was performed with 1 µg/ml puromycin. Fluorescent microscope was used for transduction efficiency assessment. After 72 h, cells were ready for RNA extraction.

Quantitative Real-Time RT-PCR Analysis

miRNAs and mRNAs were extracted from HepG2 cell lines by using RNX™-Plus (Cinnagen, Iran) according to the manufacturer's manual with slight modifications in time and the speed of centrifugation. The quality of the extracted RNA was evaluated by electrophoresis and spectrophotometry. We utilized M-MLV reverse transcriptase (Yekta tajhiz, Iran) for complementary DNA (cDNA) synthesis. Home-brew designed RT stem-loop primers were recruited for miRNA cDNA synthesis. Primers were designed using Gene Runner and AlleleID 6 software applications. Housekeeping genes, β -Actin, and small nuclear RNA (SNORD 47) were applied to normalize the expression of target mRNA and miRNA, respectively. Real-time

PCR reactions were performed in a final volume of 20 μ l, containing 0.4- μ l ROX (50 \times), 0.8 μ l of each primer (10 μ M), 10 μ l of SYBR Premix Ex Taq II 2 \times (TliRNaseH Plus, Takara, Japan), 2- μ l cDNA (<100 ng), and 6- μ l dH₂O (sterile distilled water). Non-template control was used to control any possible contamination. The real-time PCR cycling profiles were as follows: enzyme activation at 94 °C for 30 s followed by 40 cycle of denaturation at 94 °C for 5 s, and annealing/extension at 60 °C for 30 s. At the end of the amplification cycles, melting temperature analysis was set by a slow increase in temperature (0.3 °C/s) from 60 to 95 °C. The relative change in the expression of target genes was determined by applying the $\Delta\Delta$ CT method. All PCR reactions were performed on StepOne™ instrument and analyzed by StepOne™ Software v2.2.2 (Applied Biosystems, USA).

Target genes 3'-UTR Cloning in psiCHECK-2 Vectors

The sequence of 3'-UTR of *WNT1* was retrieved from NCBI, and primers were designed by using Gene Runner and AlleleID 6 software applications. The *NotI* and *XhoI* restriction enzyme sequences were added to the primer sequences in order to provide the same restriction site in the 3'-UTR of *WNT1* and the cloning vector (psiCHECK-2) for subsequent steps. PCR reactions were performed for the segment amplification with peqSTAR 96X Universal Gradient (Thermocycler United Kingdom). The PCR profile was as follows: initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 40 s, and extension at 72 °C for 90 s, followed by final extension at 72 °C for 15 min. The result was confirmed by electrophoresis. For segment purification and extraction, QIAquick gel extraction kit was used. *WNT1* 3'-UTR was first cloned in T/A vector (RBC, Korea) and then sub-cloned using *NotI* and *XhoI* restriction enzymes in psiCHECK-2 (Promega, UK) using T4 ligase. Colony PCR and restriction enzyme digestion tests were performed for confirmation.

Luciferase Assay

HEK293T cell lines were cultured in DMEM +10 % FBS, and then, seeded into 6-well plates. The cells were transfected with miR-122-containing vector and its control empty vectors pLenti III (in triplicate) separately, according to Trono lab protocols. After 48 h, cells were again transfected with psiCHECK-2 vectors, which contained 3'-UTR of the target gene *WNT1*. SV40-3'-UTR was used to detect probable off-target effect. Cells were lysed using a passive lysis buffer (Promega, Southampton, UK) and were processed with the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. Luciferase activity detection was measured using Sirius Illuminator (Berthold, Germany). The fold activation of Renilla luciferase was normalized against firefly luciferase. All of the experiments were performed in triplicate.

MTT Assay

HepG2 cell line were cultured in DMEM +5 % FBS and plated in 24-well plates at a density of 1×10^4 cells/well by adding 200 μ L of cell suspension at 5×10^4 cells. Polybrene (4 μ g/ml) was added to packaged viruses which contained miR-122 or pLenti-III. Then, HepG2 cells were transduced (in triplicate) and incubated with viruses. After 24-, 48-, and 72-h incubation, the cells were analyzed. First, cells were washed using PBS buffer to ensure that all test

compounds were removed and replaced with fresh medium. Afterwards, 100 μL of the MTT solution was added to each well and incubated for 3 h at 37 °C with 5 % CO_2 in a dark place. The MTT-containing medium was gently removed, and DMSO (200 μL per well) was added to dissolve the formed formazan crystals. Finally, the solution of each well was transferred into 2 wells in 96-well plates. The plates were read on microplate reader (Anthos2020, UK) at 570 nm.

Statistical Analysis

Real-time PCR data were analyzed using StepOne™ Software v2.2.2 by $\Delta\Delta\text{CT}$ method. Student's *t* test was used to find relationship significance between test groups in luciferase assay (SPSS 16.0), and a two-sided *p* value of less than 0.05 was considered as statistically significant. Student's *t* test also was used for MTT assay analysis.

Results

miRNA Prediction Using Bioinformatics Tools

miRNA prediction was performed using different online software applications with specific algorithms for miRNA-mRNA interaction analysis. The high-score predicted miRNAs from each software application were selected to prepare a score table. The miRNAs that were predicted in most of the applications were considered the most accurately predicted miRNAs. Thus, we selected six miRNAs which were predicted in most of the applications (Table 1). Afterwards, by using miRZ database, which is based on deep sequencing data, we obtained the predicted miRNA expression patterns to choose the best candidate for experimental analyses. The analysis of *WNT1* gene showed several miRNAs with the same score, while miR-122 had a significant reduction in HCC cells according to miRZ data. Therefore, it can be responsible for our target gene dysregulation and overexpression. Considering all these data, we concluded that miR-122 was the desired tumor suppressor miRNA for experimental analyses.

Transduction Confirmation via Fluorescent Microscope

After transduction process, we utilized fluorescent microscopy to detect green emission of cells transduced with vectors containing GFP label. More than 70 % of the HepG2 cells were transduced with miR-122-containing vector pLentiIII (Fig.1).

Expression Analysis of miR-122 in Transduced HepG2 Cells

Real-time PCR was utilized to investigate whether the viral construct was able to elevate the expression level of the predicted miRNA in HepG2 cell lines. As expected, real-time PCR results showed a logical overexpression (62-fold) in miR-122 when compared with the HepG2 cells transduced with empty control pLenti-III vector (Fig.2). miR-122 was overexpressed to prove that restoring this tumor suppressor miRNA, which was shown to be downregulated in HCC, affects the expression of *WNT1* oncogene in HepG2 cells as an HCC model.

Table 1 The result of bioinformatics prediction and scoring of miRNAs targeting 3'-UTR of WNT1

Gene name	miRNA	TargetScan	Microcosm	Pictar	miRanda	miRNAPath	Diana microT	Qiagen	miRWalk	miRZ	Sum
WNT1	miR-122	0	1	0	0	1	0	0	1	1	4
WNT1	miR-130	0	0	1	1	1	1	0	0	0	4
WNT1	miR-148	0	0	1	1	1	1	0	0	0	4
WNT1	miR-152	0	0	1	1	1	1	0	0	0	4
WNT1	miR-34	0	0	1	0	1	0	1	1	0	4
WNT1	miR-19	0	0	0	1	1	1	0	0	0	3

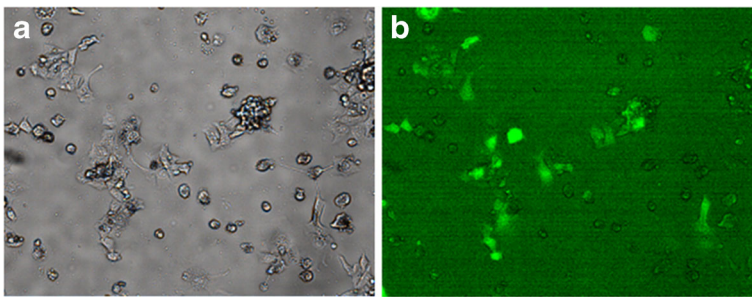


Fig. 1 HepG2 cells were transduced with miR-122-containing vector pLentiIII. **a.** Normal and **b.** green fluorescent image

Expression Analysis of *WNT1* in HepG2 Cells After Transduction with miR-122

In order to identify the effect of miR-122 transduction on *WNT1* gene, the expression of the gene was analyzed. As shown in Fig. 3, gene expression analysis showed a significant decline in *WNT1* (1250-fold) in miR-122-transduced HepG2 cells in comparison with the cells transduced with empty backbone vector pLenti-III.

WNT1 is a Direct Target of miR-122

WNT1 3'-UTR was cloned in psiCHECK-2 vector to make a construct with a specific sequence that can interact with the target miRNA. Luciferase assay was used to confirm the effect of

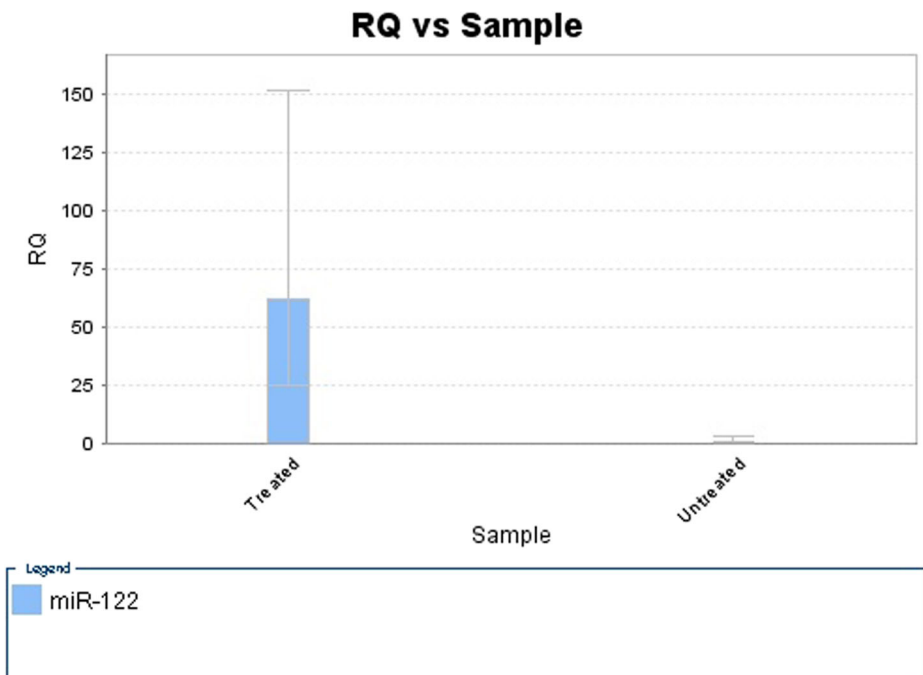


Fig. 2 miR-122 expression analysis in HepG2 cell line after transduction with miR-122-containing vector (test group) and empty vector (control group)

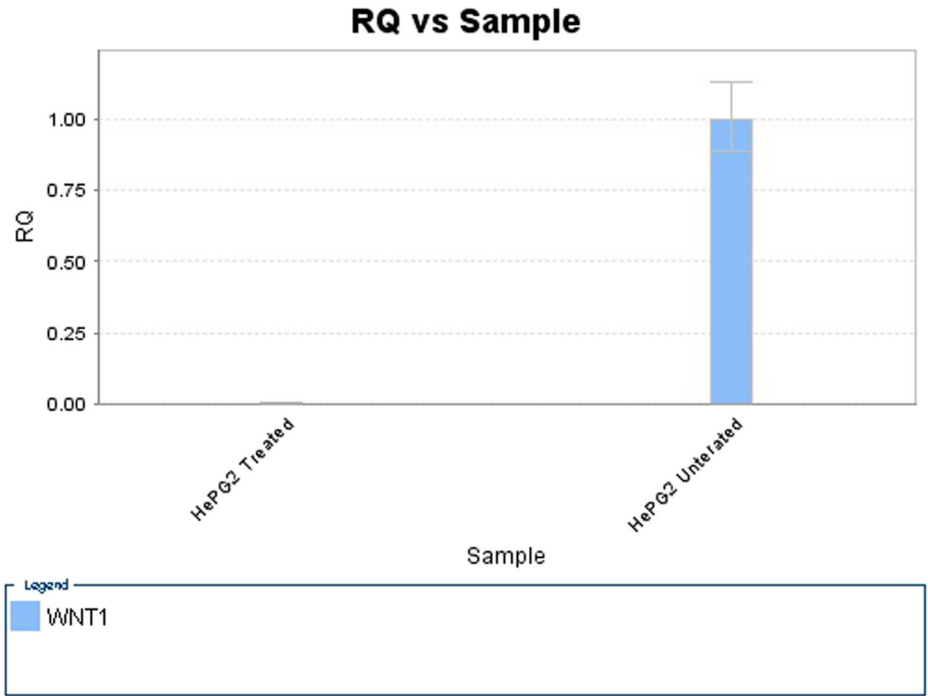


Fig. 3 *WNT1* gene expression analysis in HepG2 cell line after transduction with miR-122-containing vector (test group) and empty vector (control group)

miR-122 expression on *WNT1* 3'-UTR. Renilla (test luciferase) and firefly (internal control luciferase) fluorescent intensity were measured, and the ratio of Renilla to firefly fluorescent intensity was calculated. Student's *t* test was used to analyze the data via SPSS 16.0 software. As shown in Fig. 4, dual luciferase reporter assay verified that miR-122 regulates its targets by directly binding to its 3'-UTR. The transfection of miR-122 caused more than 2-fold decline in Renilla luciferase expression and activity in cells transfected by *WNT1* 3'-UTR containing psiCHECK-2 vector.

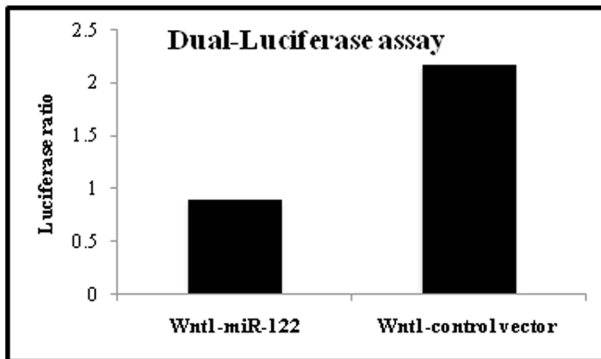


Fig. 4 Luciferase assay performed on HEK293T cell line to confirm miRNA-mRNA interaction using psiCHECK™-2 vector

The Effect of miR-122 Restoration on HepG2 Cell Viability

We showed that miR-122 transduction in HepG2 cells can lead to the elevation in miR-122 expression level. In addition, we assessed the influence of miR-122 on HepG2 cell viability. We performed MTT assay in triplicate and in two different days. After 24, 48, and 72 h of transduction, the absorbance of each well was measured at 570 nm. The results were analyzed using Student's *t* test. MTT analysis demonstrated that miR-122 restoration results in a significant decline in the number of living cells in cancer HepG2 cell line in comparison to the cells transduced by control vector (*p* value <0.05) (Fig.5).

Discussion

HCC is a lethal disease whose incidence is increasing in western countries. In spite of the discovery of new biomarkers for the diagnosis of the primary HCC and the development of new surgical methods, HCC is still highly lethal, and less than 26 % of affected individuals live more than 5 years [34]. WNT/ β -catenin signaling pathway is the key regulator of embryo as well as stem cell development, and its ectopic activation has been observed in scores of malignancies, such as HCC. This aberrant activation can be due to ectopic activation of WNT pathway genes or its ligands [24, 35]. *WNT1* is a member of *WNT* gene family. Previous studies have shown its role in cancers, and its upregulation has been observed in HCC [25, 36]. Researchers conducted by several research groups have demonstrated that some miRNAs have been dysregulated in HCC, which results in the malignant transformation of cells [25, 37–39]. Therefore, we decided to study these key regulators of *WNT1* gene, i.e., miRNAs. In a study conducted by Li et al. to find the role of miR-181b as a biomarker in atherosclerosis, researchers used TargetScan and Pictar to find the target gene [40]. Phay M et al. also used TargetScan in conjunction with miRanda for target prediction of axon-specific miRNAs in regenerating sciatic nerve [41]. In 2015, Shuming He et al. used miRWalk, DIANA-microT, and microcosm to predict the targets of miR-181b [42]. In addition, Canturk KM et al. used bioinformatics software applications to study miRNAs and key genes in bladder cancer [43]. Considering the previous studies using bioinformatics software applications and since methods such as microarray are expensive, we used bioinformatics approaches to predict miRNAs that target the desired gene.

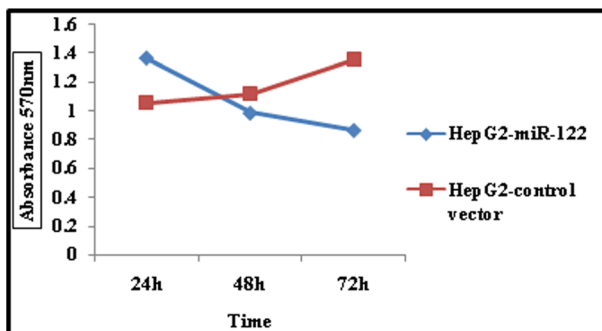


Fig. 5 HepG2 cells were transduced with miR-122 containing vector as test group and with empty vector as control vector and MTT assay were performed to measure cell viability

To improve the accuracy of the results, we used more than eight software applications. Furthermore, miRwalk and Qiagen databases were used to validate the results. Finally, the candidate miRNA was selected based on the expression profile of miRNAs provided by miRZ. The miRNA which was proven to be down-regulated in HCC was selected for further analyses.

HepG2 cells are the *in vitro* models for the study of polarized human hepatic cells. To evaluate the level of miRNA expression, RT stem-loop primers, developed by Mohammadi-Yeganeh et al. were used. This method is accurate, precise, and more importantly, cost-effective in comparison to commercial methods [44]. As expected, miR-122, which is the key regulator of cholesterol and fatty acid biosynthesis in adults' livers and shows a decline in HCC, decreased the expression of *WNT1* oncogene through targeting its 3'-UTR. The expression of *WNT1* in miR-122-transduced cells was 1250-fold less than that in the control vector-transduced cells. It is noteworthy that miR-122 expression was 62-fold higher in miR-122-transduced cells than that of control vector-transduced cells. This indicates the possible relation between the downregulation of miR-122 and upregulation of *WNT1* in HCC. To experimentally confirm this relation, i.e., direct targeting of *WNT1* 3'-UTR by miR-122, we benefited from Luciferase assay, a universal and validated assay for the evaluation of mRNA-miRNA interaction [40].

Luciferase assay results showed that miR-122 is one of the miRNAs capable of direct targeting of *WNT1* to regulate its expression. All these results confirmed the bioinformatics predictions, which showed miR-122 is a candidate miRNA that targets *WNT1*. MTT assay results of HepG2 cells transduced with miR-122-containing vector showed that overexpression of miR-122 in HepG2 cells resulted in a significant decrease of the number of viable cells. This can be interpreted by understanding the role of *WNT1*. *WNT1* promotes cell proliferation, cell cycle progression, and cellular transformation through β -catenin/Tcf4 transcription factors, and its downregulation by miR-122 results in decreased cell survival. Therefore, the knock-down of endogenous *WNT1* expression can result in cell death and can inhibit cell growth [45]. Previous studies showed that the activation of the Wnt/ β -catenin pathway by exogenous *WNT1* protects against 6-OHDA-induced changes by restoring mitochondria and endoplasmic reticulum (ER) function [46]. Furthermore, anti-*WNT1* small interfering RNA (siRNA) inhibits *WNT1* signaling, inducing apoptosis in human breast cancer MCF-7 cells [36]. Therefore, more studies are needed and recommended.

Our findings confirm the importance of *WNT1* dysregulation in HCC and also show the significant role of miR-122 on *WNT1* downregulation. Any downregulation of miR-122 tumor suppressor will result in the activation of *WNT1*. The results of our study show the capability of bioinformatics approaches in predicting and finding miRNAs and genes involved in diseases, such as cancer. It is noteworthy that the bioinformatics approach is less expensive and time-consuming than experimental approaches. Finally, considering the intriguing role of miRNAs in the prognosis and diagnosis and more optimistically, the treatment of cancer, miR-122 can be a candidate for further investigations in HCC.

Conclusion

Due to the limited therapy options, possibility of metastasis, and recurrent relapse in advanced stages of HCC, and the potential of endogenous molecules, such as miRNA in prognosis, diagnosis, and treatment of cancer, we suggest focusing on targeted therapy via miRNAs as safer and less toxic therapeutic agents. Our results approved that *WNT1* can be an excellent

candidate for further studies. Our study identifies miR-122 as a regulator of *WNT1* gene. In addition, it showed modern bioinformatics software applications and algorithms are reasonably effective with the ability to shorten the path to find cancer-gene-targeting miRNAs which are dysregulated in active signaling pathways in aggressive cancers, such as HCC.

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Compliance with Ethical Standards All the procedure of the study has been approved by Ethics Committee of the Pasteur Institute of Iran, Tehran, Iran and has been confirmed by the provisions of the Declaration of Helsinki.

Conflict of Interest The authors declare that they have no competing interests.

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