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Araştırma Makalesi - Research Article

HepG2 Hücrelerinin Kısa Uçlu β-catenin Kodlayan İfade Vektörüyle Transfeksiyonu

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ÖZ

β-katenin, Wnt sinyalleşmesinde bir efektör proteinidir. β-katenin mutasyonları; otizm, kolon kanseri, gelişimsel gecikme, zihinsel engel, nörodejenerasyon, baş, deri, ve yüz anomalileri gibi çok sayıda hastalıkların gelişmesinde raporlanmıştır. Bu hastalıklar, özellikle ekson 3 delesyonu aracılı β-katenin kısalmalarıyla ilişkilendirilmiştir. Bu nedenle β-katenin proteininin yabani tip ve ekson 3 delesyonlu formlarının fonksiyonlarını anlamak çok sayıda hastalığın tedavisinde ilerlemeyi sağlayabilecektir. Kontrollü deneyler kurmak için, yabani tip ve ekson 3 delesyonlu β-catenin formlarını kodlayan ve aynı organizmadan kökenlenen ekspresyon vektörlerine ihtiyaç duyulabilmektedir. HepG2 hücrelerinin β-katenin proteinleri bakımından heterozigot olduğu uzun zamandır bilindiği için, bu çalışmada, yabani tip ve ekson 3 delesyonlu ekspresyon vektörlerini HepG2 hücrelerinin toplam RNA'sından oluşturmanın değerli olabileceği düşünülmüştür. Bunun için, HepG2 hücrelerinden RNA izole edilmiştir, cDNA parçaları polimeraz zincir reaksiyonu (PZR) ile çoğaltılmıştır, ifade vektörleri oluşturularak 5'-uçlarından dizilenmiştir. BLAST analizi sonrası hem ekson 3 delesyonlu hem de yabani tip β-katenin kodlayan pcDNA3.1/CTNNB1 ifade vektörlerinin *E. coli* hücrelerine başarıyla klonlandığı sonucuna varılmıştır. İlginç bir şekilde, HepG2 hücreleri ekson 3 delesyonlu ifade vektörü ile transfekte edildiğinde, β-katenin protein seviyesi etkilenmemiştir. Dahası hücre morfolojisi ve populasyon ikilenme zamanı anlamlı ölçüde değişmemiştir.

Anahtar Kelimeler- Kısa uçlu β-katenin, Ekson 3, Klonlama, Wnt sinyalleşmesi, BLAST analizi

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The Transfection of HepG2 cells with Truncated β-Catenin Coding Expression Vector

ABSTRACT

β-catenin is an effector protein in Wnt signaling. β-catenin mutations are reported in the development of many diseases such as autism, colorectal carcinoma, developmental delay, intellectual disability, neurodegeneration, skin, hair and facial anomalies. Exon 3 deletion mediated truncations of the β-catenin associated with these diseases. Therefore understanding the functions of wild type and exon 3 deleted forms of β-catenin may provide an enhancement in the treatment of many diseases. However, to conduct controlled experiments, there could be a demand for the expression vectors that code for wild type and exon 3 deleted forms of β-catenin and originated from the same organism. Since it has long been known that HepG2 cells are heterozygous for β-catenin, in this study, it was found worthy of constructing the expression vectors from the total RNA of HepG2 cells. Then the utility of truncated β-catenin coding pcDNA3.1/CTNNB1 expression vector for upregulation of truncated β-catenin in HepG2 cells was examined. To this end, RNA was isolated from HepG2 cells, cDNA fragments were amplified by polymerase chain reaction (PCR), expression vectors were constructed then sequenced from 5'-prime regions. Following the BLAST analysis, it was concluded that both truncated and wild type β-catenin coding pcDNA3.1/CTNNB1 expression vectors were successfully cloned in *E. coli* cells. Interestingly, when the parental HepG2 cells were transfected with exon 3 deleted expression vector, β-catenin protein levels were not affected. Moreover, cellular morphology and population doubling time were not significantly altered.

Keywords- Truncated β-catenin, Exon 3, Cloning, Wnt signaling, BLAST analysis

I. INTRODUCTION

β-catenin, or catenin (cadherin-associated protein) beta 1, or armadillo protein, is coded from the *CTNNB1* gene. The gene is 23.2 kb in length and has 16 exons. However, the first exon does not code for any peptide. The mature CTNNB1 mRNA is 3362 nucleotides in length and 2343 nucleotides reside in the open reading frame. β-catenin protein consists of 781 amino acids [1]. Phosphorylation from N-term of β-catenin protein with the activities of the glycogen synthase kinase -3β (GSK-3β) results in the degradation of β-catenin and reduces the concentration of the protein by the ubiquitin-proteasome pathway [2, 3]. β-catenin turnover rate is controlled by the interaction with the destruction complex, which consists of Adenomatous Polyposis Coli (APC), Axin and GSK-3β.

β-catenin is mainly localized in the cytoplasm; however, wnt ligands cause the accumulation of cytoplasmic β-catenin and the accumulated proteins further translocate to the nucleus. In the nucleus, β-catenin establishes heteromeric transcription factor complexes with the T cell factor-lymphoid enhancer factor family proteins [4]. These complexes have been assumed to activate oncogenes, which can promote cell proliferation and inhibit apoptosis [5-10].

β-catenin mutations are observed in the development many diseases such as autism [11], colorectal carcinoma [12], developmental delay, neurodegeneration, hair, skin, facial anomalies [13] and intellectual disability [14]. Exon 3 mutations are of the particular interest in the ongoing researches. Indeed, increased cellular proliferation, enhanced immunosuppression by the exon 3 mutation of β-catenin are reviewed [15]. Therefore understanding the functions of wild type and exon 3 mutated forms of β-catenin could open new avenues in the treatment of many diseases.

Researchers may need the wild type and truncated forms of β-catenin coding vectors in order to observe the impacts of two different forms of β-catenin proteins in the controlled experiments. Since it has long been known that HepG2 cells express both truncated and wild type forms of β-catenin [16], it was predicted in this study that the use of total RNA which was isolated from HepG2 cells could facilitate the simultaneous construction of truncated and wild type β-catenin coding vectors. For this purpose, total RNA was isolated from HepG2 cells and converted to the cDNA, then CTNNB1 cDNAs were amplified with PCR, next expression vectors were constructed, after expression vectors cloned in the *E. coli* cells, further truncated and wild type pcDNA3.1/CTNNB1 expression vectors were sequenced, subsequently similarity of the sequences were analysed in this study. Moreover, the use of truncated β-catenin coding expression vector for altering the expression of β-catenin was examined in HepG2 cells.

II. MATERIAL AND METHOD

A. *Cell Culture*

HepG2 hepatocellular carcinoma cells (ATCC, HB-8065) obtained from Prof. Dr Hülya Sivas (Eskişehir Technical University, Eskişehir, Turkey) were cultured as a monolayer in DMEM (Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum and 1% penicillin (100 U/ml)-streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37 °C. Cells were detached from the culture flasks with the treatment of 0.25% trypsin/EDTA (Invitrogen, USA).

B. *RNA Preparation and cDNA Synthesis*

Total RNA was extracted from HepG2 cells with the use of RNeasy Mini kit (Qiagen, Hilden, Germany) according to the directions of the manufacturer. The quantity and purity of isolated RNAs were analyzed with the NanoDrop (Thermo, Wilmington, USA) device. Next, 500 ng total RNA was converted to cDNA with ProtoScript II First Strand cDNA Synthesis kit (NEB, USA) according to the manufacturers' protocol.

C. *Primer Design*

To clone truncated and wild type forms of mature CTNNB1 mRNAs from HepG2 cells, CTNNB1 mRNA sequence (NM_001098209.1) was gathered from the National Center for Biotechnology Information

(NCBI) database. Coding regions containing start and stop codons of mature CTNNB1 mRNA were selected to design the primers. By using the primer-blast program from the NCBI database, the primer pair was designed. Next, the NheI restriction site and Kozak sequence were added to the CTNNB1 forward primer, then the KpnI restriction site was added to the reverse primer. The resulting forward and reverse primers were 5'-TAATGCTAGCCACCATGGCTACTCAAGCTGATTTG-3' and 5'-CTCAGGTACCGCCCTCTCAGCAACTCTACA-3', respectively.

D. PCR Amplification of cDNA Fragment

Polymerase chain reaction (PCR) was performed in a 50 µl mixture containing 25 µl of 2× Q5 high fidelity master mix (NEB, USA) with Mg^{++}, 1 µl of mixed dNTPs (0.2 µM each), 2.5 µl of each specific primer (0.5 10 µM), 0.5 µl of Q5 high fidelity DNA polymerase (1 u/µl) (NEB, USA), 4 µl of template cDNA and 17 µl of water. The PCR program was 98 °C for 30 seconds, then 30 cycles of 98 °C for 10 seconds, 63°C for 20 seconds and 72 °C for 20 seconds, followed by 72 °C for 2 minutes. In the end, the PCR products were run on 1.5% agarose electrophoresis.

E. Construction of the Expression Vectors

The PCR products were run on 1% agarose gel electrophoresis then the DNA bands were cleaned by QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the guidelines of the manufacturer. The purified PCR products were inserted into the NheI and KpnI sites of the pcDNA3.1/Hygro (Invitrogen, USA) expression vector (Invitrogen, USA) by double digestion with corresponding enzymes. Next, organic extraction with phenol: chloroform was implemented to eliminate the restriction enzymes from the solution containing digested PCR products and expression vector as previously indicated [17]. Then, colon purification was performed with Chromaspin TE-1000 (Clontech, Heidelberg, Germany) to eliminate the contaminating oligonucleotides, salts and other chemicals further. After the concentrations of purified PCR products and expression vector were assessed by NanoDrop (Thermo, Wilmington, USA) device and the lengths of digested PCR products and expression vector were analysed by 1.5% agarose electrophoresis. The ligation reaction was set up with combining 10 ng vector and 30 ng PCR products in 1× T4 DNA ligation buffer (NEB, USA).

F. Transformation, Selection and Validation of Recombinant E.coli Cells

The products of the ligation reaction were transformed into E.coli DH5-α cells. Next, transformed E.coli cells were selected in plates of 100 µg/ml Ampicillin containing Luria-broth (LB/Amp) medium. Colony PCR was performed to validate the truncated and wild type CTNNB1 inserts in the vectors.

G. Sequencing of Truncated and Wild Type pcDNA3.1/CTNNB1 Expression Vectors

Positive colonies were further incubated in LB/Amp medium and plasmid isolation was carried out using Nucleospin miniprep (Macherey-Nagel, Düren, Germany) with the instructions of the manufacturer. The purity and quantity of isolated plasmids were evaluated with NanoDrop (Thermo, Wilmington, USA) device. Next, Sanger sequencing was performed by using CMV-forward primer in ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA) which is available in a company (Sentegen, Ankara, Turkey).

H. Analysis of Sequence Similarity

Database sequence similarity search in the Basic Local Alignment Search Tool (BLAST) program was performed at the NCBI database to find out the sequences that correspond to the truncated and wild type pcDNA3.1/CTNNB1 expression vector sequencing data.

İ. Stable Transfections and in-Cell Western Analysis

Transfections with truncated pcDNA3.1/CTNNB1 expression vector and the empty pcDNA3.1/Hygro plasmid were performed by using Turbofect Reagent (Thermo, Germany) as previously described [18]. In order to examine whether the β-catenin levels with the transfection of truncated pcDNA3.1/CTNNB1 expression vector in HepG2 cells, the in-cell Western analysis was performed as previously described [18]. Briefly, 5,000

cells were seeded to each well of 96-plate and incubated for 48 h. Next the cells were fixed with ice-cold methanol for 10 minutes at -20 °C. Then the cells were washed with phosphate buffered saline (PBS). After the cells were blocked in Odyssey blocking buffer (Licor, Lincoln, USA). Further the cells were incubated with an anti-β-catenin antibody (CST, Danvers, USA) in 0.1% Tween-20 containing Odyssey blocking buffer for overnight at 4 °C in a shaker. Next the cells were washed with PBS/0.1% Tween-20. Then cells were incubated with an Infrared IRDye800CW secondary antibody (Licor, Lincoln, USA) and cell tag stain (Licor, Lincoln, USA) in 0.2% Tween-20 containing Odyssey blocking buffer for 1 h in room temprature. Subsequently cells were washed with PBS and imaged on an infrared scanner (Odyssey, Licor, Lincoln, USA) using both 700 and 800 nm wavelength channels. The signals from 800 nm wavelength were used for quantification of β-catenin expression. The signals from 700 nm wavelength were used for quantification of cell number. Data were analysed with ImageStudio tool (Licor, Lincoln, USA).

J. Morphological Observations

Alterations in the morphology of HepG2-tCTNNB1 cells which were transfected with pcDNA3.1/CTNNB1and HepG2-EV cells which were transfected with pcDNA3.1/Hygro were assessed by an inverted microscope (BX 50, Olympus, USA).

K. Population Doubling Time Analysis

For the analysis of population doubling times, 3×10^6 cells were seeded into 8.5 cm^2 petri dishes. The number of cells per dish was counted every day for 3 days with a Cedex (Roche Innovatis, Germany) cell counter. The population doubling time of HepG2-tCTNNB1 and HepG2-EV cells were calculated with the formula of "Population doubling time=Culture period in hours*In2/[In(Number of cells T=O/Number of cells T=1)]".

L. Statistical Analysis

Statistical analyses, graphing were implemented by using GraphPad (GraphPad Prism 6, San Diego, CA, USA). Student t-test was used for differential analyses and p-values less than 0.05 were considered to be statistically significant.

III. RESULTS AND DISCUSSION

Truncated and wild type forms of CTNNB1 coding mRNAs that are originated from HepG2 cells were cloned in *E. coli* cells then sequenced in this research. The cloning strategy was consisting of the amplification of truncated and wild type forms of CTNNB1 cDNAs with the same primer pair that is mentioned in the material methods section. Next, the amplified PCR products were inserted to the NheI/KpnI sites of the pcDNA3.1/Hygro mammalian expression vector. Then the inserts were checked on the isolated vectors by the digestion with NheI enzyme and run on standard 1.5% agarose gels stained with ethidium bromide. As is shown in Figure 1, Lambda DNA-BstE II digest (NEB, USA) which is the standard ladder was loaded to the first lane. Digested pcDNA3.1 expression vectors, in which the inserts were truncated and wild type forms of CTNNB1 cDNAs, were loaded to the second lane. Digested pcDNA3.1 expression vectors, in which the inserts were truncated form of CTNNB1 cDNAs, were loaded to the third and fourth lanes. Digested pcDNA3.1 expression vector, in which the insert was wild type form of CTNNB1 cDNA, was loaded to the fifth lane. Based on the observations from Figure 1, it was concluded that the cloning experiment resulted in the construction of two different expression vectors that are different about 300-400 nucleotides in their size. In order to validate that these vectors code different forms of β-catenin, the sequencing experiment was conducted. Then the resulting sequences were analyzed with the BLAST program to find out the similar sequences in the reference RNA database.

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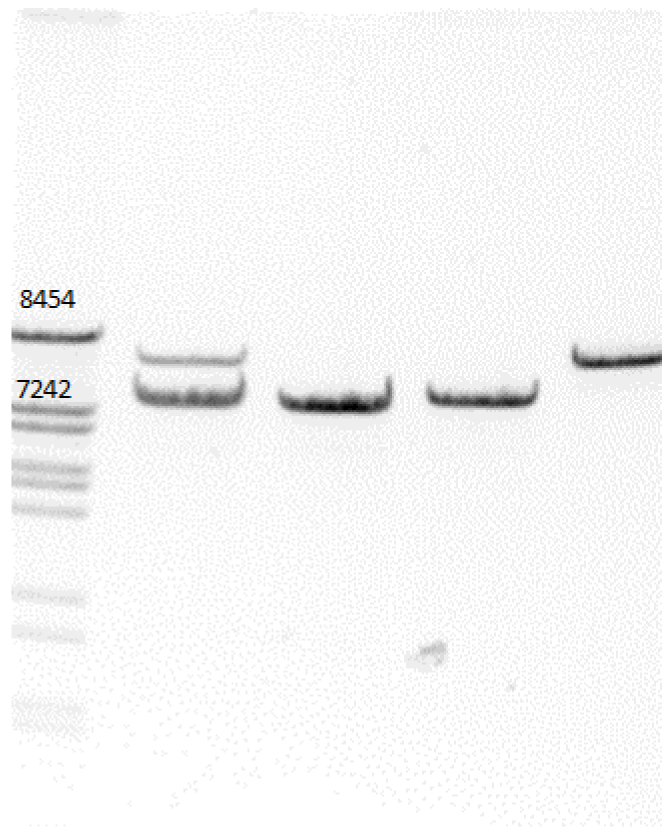

Figure 1. Validation of existence of the truncated and wild type forms of CTNNB1 cDNAs in the pcDNA3.1 expression vectors that were digested with NheI enzyme and run on standard 1.5% agarose gel, which was stained with ethidium bromide. Lambda DNA-BstE II digest (NEB, USA) standard ladder is in the first lane. pcDNA3.1 expression vectors coding for the truncated and wild type forms of β-catenin are in the second lane. pcDNA3.1 expression vectors coding for the truncated form of β-catenin is in the third and fourth lanes. pcDNA3.1 expression vector coding for the wild type β-catenin is in the fifth lane

It was observed from the BLAST reports (Figure 2) that the insert in the pcDNA3.1/CTNNB1 vector was 99% homolog to the CTNNB1 reference RNA sequence (NM_001098209.1). In addition, the insert in the truncated pcDNA3.1/CTNNB1 vector was also 98% homolog to the CTNNB1 reference RNA sequence. Truncated pcDNA3.1/CTNNB1 vector has a deletion that is 348 nucleotides in length, which corresponds to 286-635 nucleotides in the reference RNA. Since this deletion may result in partial incorporation of exon 3 and exon 4, the translated β-catenin protein will be deprived of 116 amino acids. In the literature, this deletion has been associated with the accumulation of β-catenin, because the deleted site in the truncated β-catenin is responsible for the phosphorylation executed by GSK‑3β. Moreover, N-terminal β-catenin phosphorylations by GSK‑3β promote the turnover rate of β-catenin [16].

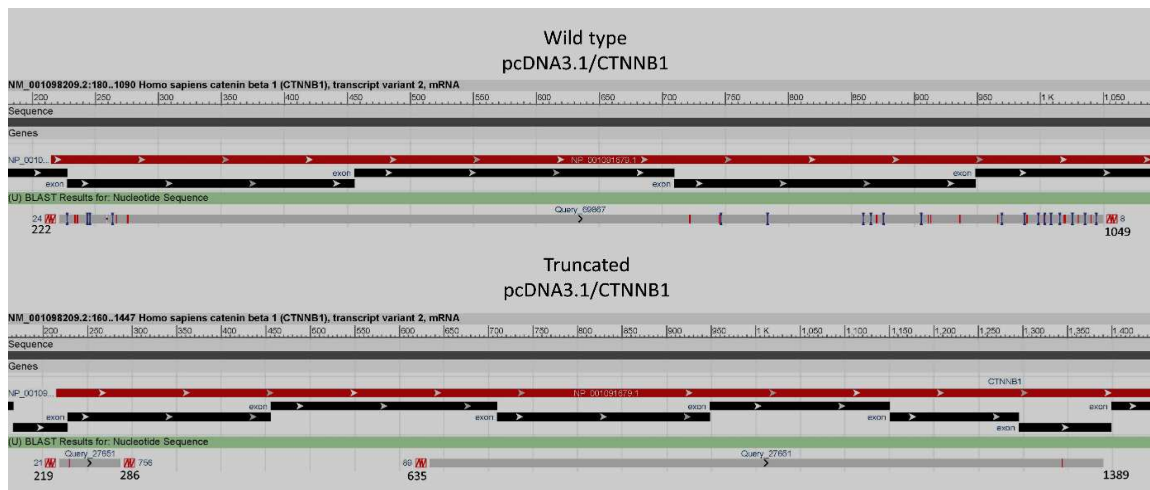

Figure 2. Blast reports of wild type and truncated pcDNA3.1 vectors. Red lines in the figure stand for reference RNA sequence, black lines for exons and the grey lines for query sequences that were subjected to BLAST analysis. The sequenced part of wild type pcDNA3.1/CTNNB1 vector corresponds to the nucleotides between 222-1049 in the reference RNA (NM_001098209.1). The sequenced part of the truncated pcDNA3.1/CTNNB1 vector corresponds to the nucleotides between 219-286 and 635-1389 in the reference RNA (NM_001098209.1). Since truncated pcDNA3.1/CTNNB1 vector has a deletion of 348 nucleotides in length, exon 3 and exon 4 is partially incorporated into the protein.

Then the parental HepG2 cells were transfected with truncated pcDNA3.1/CTNNB1 vector and resulting cells were growth under the antibiotic pressure. Next, the cells were investigated for the β-catenin expression. It was observed from the in-cell western analysis that relative β-catenin levels were not significantly different in HepG2/EV and HepG2/tCTNNB1 cells (Figure 3). In the literature, it was reported that β-catenin lacking its C terminus failed to accumulate in *Drosophila melanogaster* [19]. In another study, the authors suggested that the flexible C-terminal region of β-catenin is intrinsically required for its stabilization [20]. In this study, the reason for the stable levels of β-catenin by the transfection with truncated pcDNA3.1/CTNNB1 expression vector in HepG2 cells could not be related to the poorer stabilization of truncated β-catenin by deletion of its N-terminal region. Because truncated N-terminal associated with the accumulated β-catenin [16]. Therefore, the explanation of this observation remains as a question for further studies.

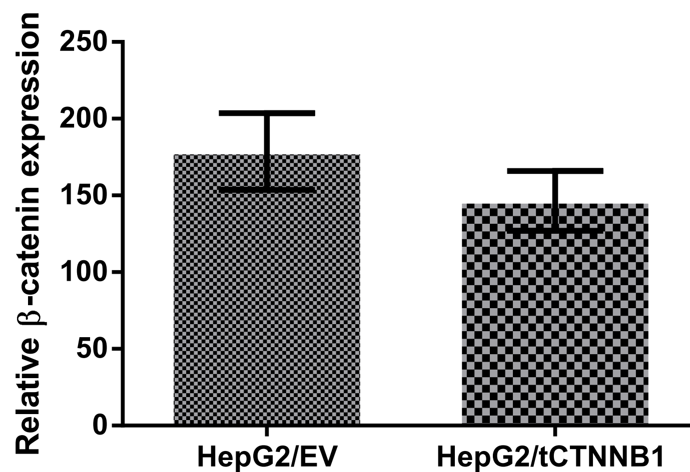

Figure 3. Transfection with truncated pcDNA3.1/CTNNB1 expression vector was not significantly altered the β-catenin levels in HepG2 cells. Relative β-catenin expressions, which were determined by in-cell western analysis, were not significantly different in HepG2/EV and HepG2/tCTNNB1 cells ($p>0.05$).

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Morphological analysis of HepG2-EV and HepG2-tCTNNB1 revealed that transfection with pcDNA3.1/Hygro and truncated pcDNA3.1/CTNNB1 expression vector was not significantly altered the morphology of HepG2 cells (Figure 4).

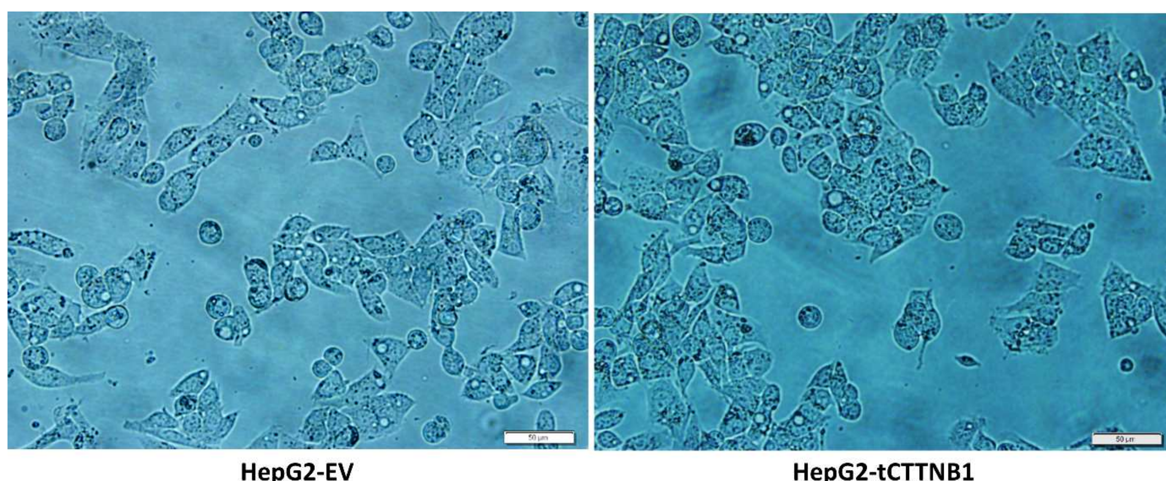

Figure 4. Morphological comparison of HepG2-EV and HepG2-tCTNNB1 cells indicated that these two cells were not significantly different from each other. Scale bars are 50 µm

As indicated in Table 1 population doubling times were 26.03 and 26.56 for HepG2-EV and HepG2-tCTNNB1, respectively. The population doubling times of HepG2-EV and HepG2-tCTNNB1 cells were not significantly different. Therefore, transfection with pcDNA3.1/Hygro and truncated pcDNA3.1/CTNNB1 expression vector was not significantly altered the population doubling times.

Table 1. Population doubling times for HepG2-EV and HepG2-tCTNNB1 cells. Data are shown as Mean ± SEM of three independent biological replicates.

Cell Line	Population Doubling Time (h)
HepG2-EV	26.03±2.9
HepG2-tCTNNB1	26.56±3.0

IV. CONCLUSION

In this research, two different forms of CTNNB1 coding cDNAs were simultaneously inserted into the pcDNA3.1 vector. Since β-catenin is an essential mediator of cell-cell adhesion and a transcription factor in the Wnt pathway, this cloning experiment can be considered as an initial step for conducting further experiments that aim to understand the different functions of truncated and wild type β-catenin proteins in the cell. In addition, transfection with truncated pcDNA3.1/CTNNB1 expression vector was not altered the β-catenin levels, morphology and population doubling times in HepG2 cells. Therefore it is not a good idea to upregulate the expression of the truncated form of β-catenin by using truncated pcDNA3.1/CTNNB1 expression vector in HepG2 cells.

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