

A role of functional T-type Ca^{2+} channel in hepatocellular carcinoma cell proliferation

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Abstract. The role of T-type Ca^{2+} channels in hepatocellular carcinoma cell proliferation was investigated *in vitro*. Eleven hepatocellular carcinoma cell lines and one immortalized liver cell line (LO2) were examined for the status of T-type Ca^{2+} channels with RT-PCR and voltage-clamp recordings. Except HBx344, other cell lines tested had one, two or all three of $\alpha 1$ -subunits ($\alpha 1\text{G}$, $\alpha 1\text{H}$ and $\alpha 1\text{I}$) mRNA expression. Obvious T-type current was recorded in SNU449 cells, while others exhibited a minimal or no T-type current. SNU449, PLC/PRF5, Hep3B and LO2 cell lines were subjected to growth assay in the presence of Mibefradil, a T-type Ca^{2+} channel blocker, only the proliferation of SNU449 cell which had functional T-type Ca^{2+} channel was reduced by Mibefradil treatment. Furthermore, the persistent increase of phosphorylated ERK1/2 in SNU449 cells was found when treated with Mibefradil. A microarray assay also demonstrated some down-regulated genes were mainly associated with cell cycle and cell proliferation in Mibefradil treated SNU449 cells. In conclusion, this study showed that the functional T-type Ca^{2+} channels probably participate in modulating the proliferation of some hepatocellular carcinoma cells. The cell proliferation reduction of SNU449 with Mibefradil treatment is possibly associated with the persistent increase of phosphorylated ERK1/2.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide, and more than a half of HCC new cases occur in China (1). HCC is a very heterogeneous disease in terms of its etiology, molecular tumorigenesis mechanisms and biological behavior. Although the pathogenesis of HCC remains incompletely understood, it is believed to involve several sequential events, including chronic inflammation, hepatocyte hyperplasia and ultimately malignant transformation (2). In order to understand the molecular mechanism of tumor cell proliferation and explore the potential targets for therapy, many studies have been performed to identify aberrant signaling pathways for initiating and promoting the growth of HCC, especially the key switch on controlling the unlimited proliferation of tumor cells.

Ca^{2+} , as an intracellular signal, is involved in many different cell processes, including membrane excitability, synaptic transmission, activation of enzymes and regulation of gene expression. It also plays important roles in cell cycle progression, cell apoptosis, differentiation and proliferation (3). For example, Ca^{2+} is an important growth signal for cells, and its expression implicates the proliferation of cells (4). Its role in proliferating non-tumorous tissue has been reported in vascular smooth muscle (5) and newborn rat cardiomyocyte (6). Furthermore, some studies have reported its role in the cell proliferation of brain tumors (7), prostate tumors (8) and retinoblastoma (9). In retinoblastoma cells, the decreased proliferation is accompanied by the decreased expression of mRNA for T-type Ca^{2+} channels and the decreased T-type Ca^{2+} currents.

One study has reported that the disruption of Ca^{2+} mobilization in cells that is normally growth-inhibited by cAMP can derepress the B-Raf/ERK pathway, and further convert these cells to a phenotype with growth-stimulated by cAMP (10). Therefore, Ca^{2+} homeostasis inside the cells is related to normal growth of the cells. Uncontrolled proliferation is a prominent feature in cancer cells, and several studies have shown T-type Ca^{2+} channel is associated with the proliferation of cancer cells (11,12). Better understanding of the molecular mechanism of the T-type Ca^{2+} channels in cancer

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Table I. Sequence-specific primers for three T-type Ca^{2+} subunits ($\alpha 1\text{G}$, $\alpha 1\text{H}$, $\alpha 1\text{I}$).

Subunits	Forward primer	Reverse primer	Product size (bp)
$\alpha 1\text{G}$	5'-GCTCTTTGGAGACCTGGAGTGT-3'	5'-TAGGCGAGATGACCGTGTTG-3'	197
$\alpha 1\text{H}$	5'-TTGGGTTCCGTCGGTTCT-3'	5'-ATGCCCGTAGCCATCTTCA-3'	193
$\alpha 1\text{I}$	5'-ATCGGTTATGCTTGGATTGTCA-3'	5'-TGCTCCCGTTGCTTGGTCTC-3'	200

cell proliferation will have an important significance for clinical therapies. In the present study, an association between the functional T-type Ca^{2+} channel and the proliferation of HCC cells was investigated for the first time.

Materials and methods

Cell culture. Eleven HCC cell lines (Hep3B, HepG2, HBx F344, HuH-1, PLC/PRF5, SKHep1, SMMC7721, SNU182, SNU387, SNU449 and SNU475) and one immortalized liver cell line (LO2) were investigated in this study. All cell lines were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (Gibco, USA). Cultured cells were maintained at 37°C in a humidified incubator with 5% CO_2 in the air.

T-type Ca^{2+} channel subunits detected by RT-PCR. Total RNA was isolated from each HCC cell line with TRIzol reagent (Invitrogen, USA), and cDNA was produced from total RNA with oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). All the operations were performed according to manufacturer's protocols. The cDNA product was used as a template for subsequent PCR amplifications for $\alpha 1\text{G}$, $\alpha 1\text{H}$ and $\alpha 1\text{I}$ subunits. The forward primer for GAPDH was 5'-GAGTCAACGGATTTGGTCGT-3'. The reverse primer for GAPDH was 5'-TTGATTTTGGAGGGA TCTCG-3'. Sequence-specific primers of T-type Ca^{2+} channel subunits are shown in Table I.

Cellular electrophysiology analysis. Patch-clamp recordings of Hep3B, HuH-1, PLC/PRF5, SNU449, SMMC7721 and LO2 were performed in the whole-cell configuration as previously described (13). The currents were evoked by either step depolarization or two-step voltage protocol. The whole experiment was performed at room temperature.

Cytotoxicity assay. A cytotoxic assay of Mibefradil (Sigma, USA) on SNU449 with different concentrations (0, 2.0, 3.0, 4.5, 5.0 and 6.0 $\mu\text{mol/l}$) was performed. Firstly, SNU449 cells were cultured in 6-well plates, and treated with different concentrations of Mibefradil for 72 h, and then the cells were washed with the phosphate-buffered saline (PBS) twice, and harvested with the trypsin. Finally Live/Dead® Viability/Cytotoxicity reagents (Invitrogen) were added to the suspended cells in PBS according to manufacturer's instructions, and assayed with the flow cytometer.

Cell proliferation assay. HCC cells were seeded in 96-well culture plates at a density of 2×10^3 cells per well. Culture

media (120 μl) containing 3.0 $\mu\text{mol/l}$ of Mibefradil was added to the 96-well plates after the cells were attached. Then the number of living cells was measured every day for up to 7 days. MTT (12 μl) (5 mg/ml, Amerisco, USA) was added to each well, and the plates were incubated at 37°C for 4 h. The culture medium in the 96-well plates was discarded, and then 150 μl of DMSO was added to the wells and mixed gently. After 15 min, the optical density (OD) was read on a microplate autoreader (Bio-Rad Model 680 Microplate Reader, USA) at 490 nm. Each assay was performed in triplicate.

shRNA targeting T-type Ca^{2+} channel $\alpha 1\text{G}$ gene treatment. Five lentiviral-based shRNA constructs targeting different regions of human $\alpha 1\text{G}$ gene had already been obtained (11). Transduction of shRNA into the HCC cells was achieved by a lentiviral infection method. The recombinant lentivirus was generated by using a phoenix packaging cell line, plasmids and Lipofectamine™ 2000 reagent (Invitrogen). Lentiviral supernatant was collected at 48 h after transfection of lentiviral vector. The collected lentiviral supernatants were finally pooled and filtered through a 0.45- μm filter, and then were used to treat the SNU449 cells.

Stable transfection experiment. HepG2 without endogenous T-type Ca^{2+} channel $\alpha 1\text{G}$ subunit expression was transfected with an expression plasmid containing human $\alpha 1\text{G}$ gene (pcDNA3.1 $\alpha 1\text{G}$). Firstly, HepG2 cells were seeded in 60-mm dishes until 70% confluence, and then the cells were transfected with pcDNA3.1 $\alpha 1\text{G}$ or the pcDNA3 empty vector as a control with Lipofectamine 2000 reagent. Transfectants were selected in the complete medium containing 0.4 mg/ml of G418 sulfate (Inalco, USA). After two weeks, individual colonies were harvested and expanded. Total RNA was isolated from the HepG2 cells transfected with pcDNA3.1 $\alpha 1\text{G}$, and T-type Ca^{2+} channel $\alpha 1\text{G}$ subunit was detected by RT-PCR.

Microarray analysis. Duplicate cultures of SNU449 cells were seeded in 100-mm dishes at density 5×10^5 cells and treated with or without 4.5 $\mu\text{mol/l}$ of Mibefradil for 72 h. This concentration had no toxicity on SNU449 cells. T-type Ca^{2+} channel (80%) was blocked when cells were treated with this concentration. Total RNA was isolated from each of SNU449 cell samples. Appropriate RNA was used as a template for cDNA synthesis on PCR Thermo-cycler (GeneAmp 9700). About 15 μg of Cy3-cDNA and 15 μg of Cy5-cDNA samples were hybridized to a Human Whole Genome OneArray™ Genechip Array (Phalanx Biotech Co., Taiwan) in hybridization oven for 16 h at 42°C. After hybri-

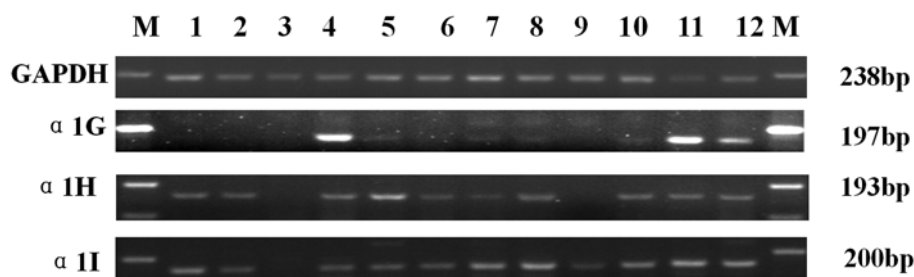


Figure 1. Expression of T-type Ca^{2+} channel α_1 -subunits identified by RT-PCR amplification in cell lines. M, DNA marker; Lane 1, Hep3B; Lane 2, HepG2; Lane 3, HBx/F344; Lane 4, HuH-1; Lane 5, PLC/PRF5; Lane 6, SKHep1; Lane 7, SMMC7721; Lane 8, SNU182; Lane 9, SNU387; Lane 10, SNU449; Lane 11, SNU475; Lane 12, LO2.

dization, each array was visualized with an Axon GenePix 4000B scanner.

GenePix™ 4100 software was used to extract data from Axon GenePix 4000B scanner. The hybridized array signal was expressed as fluorescence intensity. The gene expression profiles of Mibefradil treated and untreated SNU449 cells were analyzed based on 1.4-fold Log2 ratio in fluorescence intensity. The genes affected by Mibefradil were analyzed on <http://david.abcc.ncifcrf.gov/>.

Western blotting analysis. Total 2×10^5 cells were plated in 60-mm dishes. After 24 h, the cells were cultured in control medium or medium with concentration gradient of Mibefradil, and incubated for different time period. The cells were rinsed with PBS for twice and lysed in 200 μl of ice-cold lysis buffer (Beyotime, China). Insoluble cell lysate was removed by centrifugation at 13000 rpm for 15 min at 4°C . Concentration of soluble cell protein was measured by BCA protein assay kit (Thermo Fisher Scientific, USA). Lysate proteins (40 $\mu\text{g}/\text{lane}$) were heated at 95°C in SDS buffer for 5 min, and then were separated on a 10% SDS-PAGE. The proteins were electroblotted to nitrocellulose membranes. The membranes were blocked with the 5% defatted powdered milk in TBST [10 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 0.05% Tween-20] for 1 h at room temperature. The blocked membranes were incubated with antibodies (anti-cyclin D1, Invitrogen; anti-ERK1/2 and anti-pERK1/2, Santa Cruz, USA; anti-p21 and anti-tubulin, Beyotime) diluted in the above buffer for 2 h at room temperature. Then, the membranes were washed three times with TBST and incubated with IRDye800CW-IgG secondary antibodies (Licor, USA) for another 1 h at room temperature. The membranes were again washed three times with TBST. Licor scanner and Odyssey v1.2 software (Licor) were used for scanning the bands.

Data analysis. Statistical analysis was performed using two side unpaired t-test for comparisons between two groups, and P-value <0.05 was considered to be significant. GraphPad Prism® version 4.0 software was used for data analysis.

Results

Expression of T-type Ca^{2+} channel α_1 -subunits in cell lines. Six cell lines (HuH-1, PLC/PRF5, SMMC7721, SNU182, SNU449 and SNU475) expressed all three T-type α_1 -subunits.

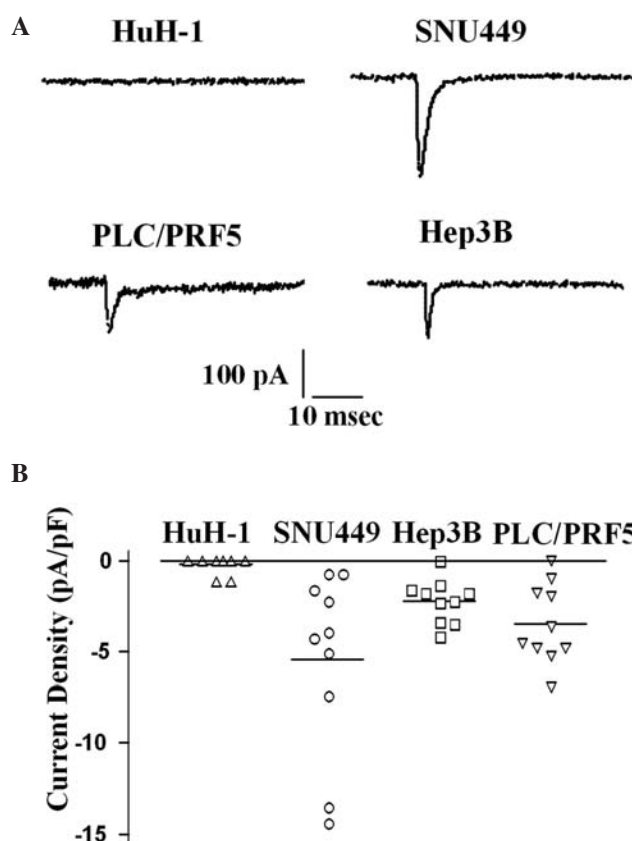


Figure 2. Functional expression of T-type Ca^{2+} channels in the HCC cells. (A) Representative deactivating tail current traces in SNU449, PLC/PRF5 and Hep3B. No current was detected in HuH-1 cells. (B) Individual data points of maximally elicited T-type tail currents in HuH-1, SNU449, Hep3B and PLC/PRF5 cells. Data represent the mean \pm SEM of four independent experiments.

Three cell lines (Hep3B, HepG2 and SKHep1) expressed $\alpha_1\text{H}$ and $\alpha_1\text{I}$, SNU387 only expressed $\alpha_1\text{I}$. No T-type α_1 -subunit expression was observed in HBx/F344 while LO2 expressed three T-type α_1 -subunits (Fig. 1).

Functional expression of T-type Ca^{2+} channels in cell lines. SNU449 cells exhibited a more significant T-type current among the tested cell lines, PLC/PRF5 and Hep3B showed minimal current, and HuH-1 had no current (Fig. 2). SMMC7721 also showed minimal current, and no current was observed in LO2 either (data not shown).

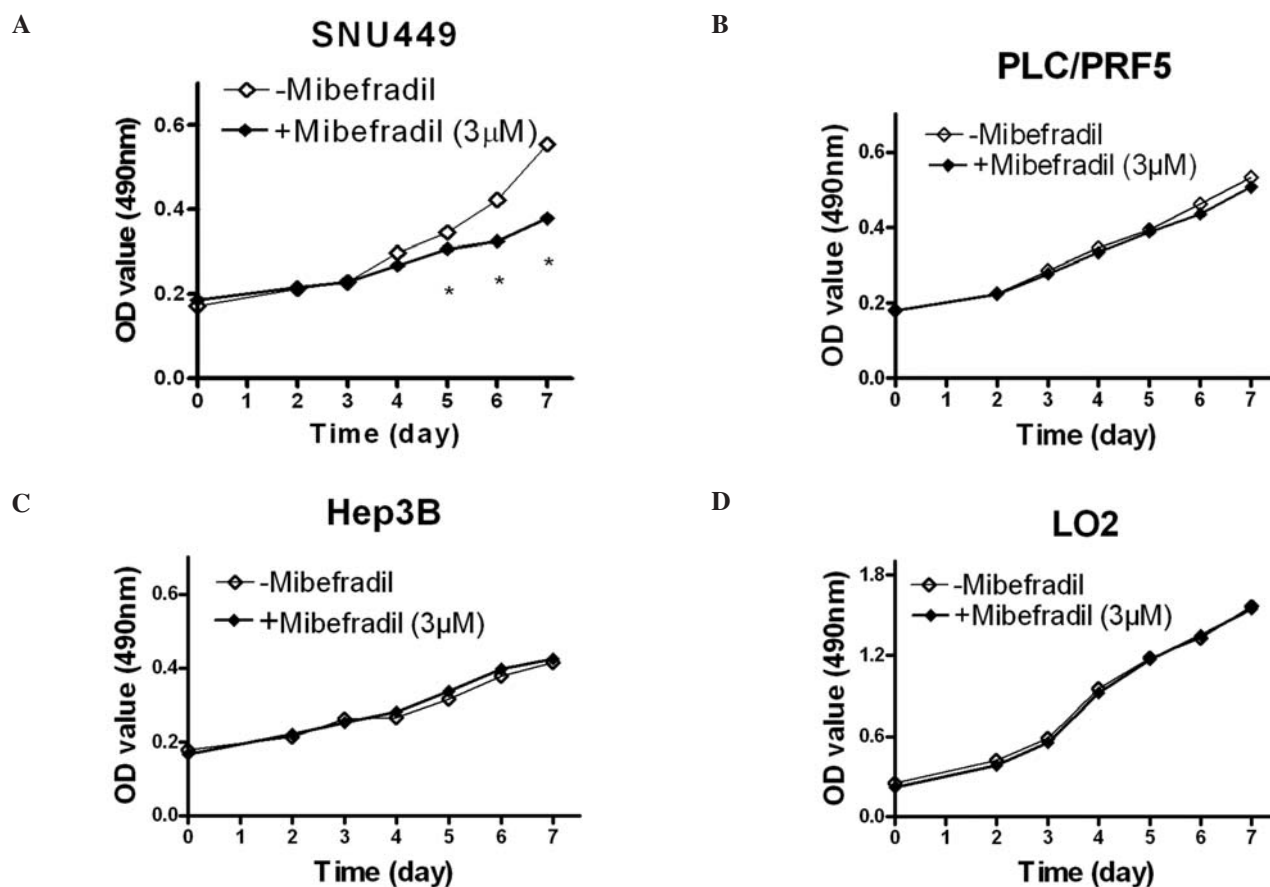


Figure 3. Effect of T-type Ca^{2+} channel blocker Mibefradil on cell proliferation. Cell growth was observed by MTT growth curve over a 7-day period with and without Mibefradil ($3.0 \mu\text{mol/l}$) in SNU449 (A), PLC/PRF5 (B), Hep3B (C) and LO2 (D) cells. Mibefradil reduced the proliferation of SNU449 cells ($^*P < 0.05$ versus Mibefradil untreated group at the same time-points), but had no effect in PLC/PRF5, Hep3B and LO2 cells (no difference between Mibefradil-treated and untreated groups).

Inhibitor of T-type Ca^{2+} channel reduced the proliferation of HCC cells. The concentration of $3.0 \mu\text{mol/l}$ of Mibefradil used to treat the cells was an approximate IC_{75} value for all three T-type α_1 -subunits (14). It resulted in a significant decrease in the number of proliferating SNU449 cells compared to controls (Fig. 3A). The antiproliferative effect of Mibefradil on SNU449 cells was specific for T-type Ca^{2+} channel blockade since Mibefradil had no inhibitory effect on the proliferation of cell types exhibiting minimal or no functional T-type Ca^{2+} channels, such as PLC/PRF5, Hep3B with minimal functional T-type Ca^{2+} current, and LO2 cell without T-type current (Fig. 3B-D).

shRNA silencing $\alpha_1\text{G}$ subunit had no effect on cell proliferation. To determine whether T-type Ca^{2+} channel $\alpha_1\text{G}$ subunit acts as a predominant role in affecting SNU449 cell proliferation, RNAi approach was used to silence the $\alpha_1\text{G}$ gene of SNU449 cells. The effect of $\alpha_1\text{G}$ silencing on SNU449 cell proliferation was observed by the MTT assay. However, the lentiviral-based small hairpin RNA did not change the proliferation of SNU449 cells (data not shown). It indicated that $\alpha_1\text{G}$ subunit may not play a dominant role in regulating the proliferation of SNU449 cells.

Ectopic expression of $\alpha_1\text{G}$ subunit did not alter cell proliferation. In order to confirm whether T-type Ca^{2+} channel

$\alpha_1\text{G}$ subunit expression promotes the proliferation of other HCC cell lines, HepG2 cells without T-type Ca^{2+} channel $\alpha_1\text{G}$ subunit expression were transfected with a pcDNA3.1h $\alpha_1\text{G}$ vector. After G418 sulfate selection, exogenous $\alpha_1\text{G}$ subunit was successfully expressed in HepG2 transfected with pcDNA3.1h $\alpha_1\text{G}$ as confirmed by RT-PCR. However, ectopic expression of T-type Ca^{2+} channel $\alpha_1\text{G}$ subunit in HepG2 did not change the cell proliferation compared to the control, as demonstrated by MTT growth curve assay (Fig. 4).

Gene expression in SNU449 cells affected by Mibefradil treatment. To confirm which genes are regulated by the functional T-type Ca^{2+} channels and contribute to the proliferation of HCC cells, the whole genome expression profile analysis was performed by microarray approach on SNU449 cells. Based on the 1.4-fold difference in gene expression level between Mibefradil treated and untreated SNU449 cells, the expression of 244 genes was significantly affected by Mibefradil treatment. The above affected genes were mainly distributed in transport and homeostasis, response to stress and to external stimulus, regulation of phosphorylation and protein kinase activity, regulation of cell cycle, cell proliferation, and cell-cell signaling. Since uncontrolled proliferation is an important characteristic of cancer cells, those genes related to cell proliferation or cell cycle and down-regulated are shown in Table II.

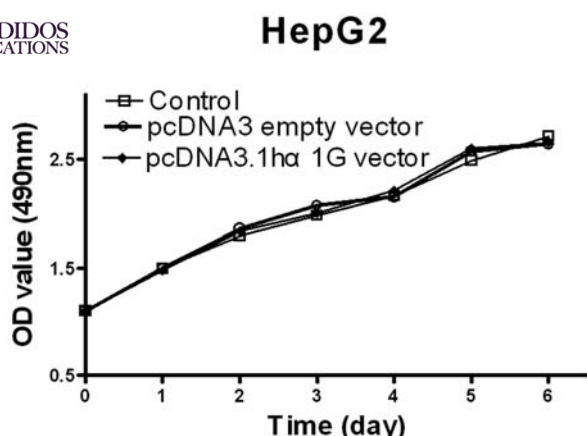


Figure 4. Effects of α 1G gene expression on HepG2 cell proliferation. Cell growth was observed over a 6-day period in normal control, pcDNA3 empty vector-transfected, and pcDNA3.1h α ₁G vector-transfected HepG2 cells. Expression of α 1G gene in HepG2 did not increase cell proliferation compared to normal control cells or cells transfected with pcDNA3 empty vector. There was no difference in cell growth in HepG2, HepG2 transfected with pcDNA3.1h α ₁G and pcDNA3.

The persistent increase of phosphorylated ERK1/2 in SNU449 cells. To study the molecular mechanism of the functional T-type Ca²⁺ channels contribution to the proliferation of HCC cells, the protein expression profiles of cyclin D1, p21, ERK1/2 and pERK1/2 were examined by blotting analysis in the HCC cells treated with 4.5 μ mol/l of Mibefradil at different time-points. The results showed that the expression levels of cyclin D1 and p21 were not significantly different between Mibefradil treated and untreated SNU449 cells and LO2 cells. However, a persistent increase of phosphorylated ERK1/2 in SNU449 cells treated with Mibefradil was observed (Fig. 5). Furthermore, we found that Mibefradil induced the sustained increase of pERK1/2 in a concentration-dependent manner.

Discussion

Although several studies had shown T-type Ca²⁺ channels played a role in the proliferation of many tumor cells, the

Table II. Genes regulated by Mibefradil in SNU449 cells.

Functional group ^a Gene name	Gene symbol	Gene expression changed by Mibefradil treatment vs. control
Phosphatidylinositol signaling system		
CDC-like kinase 4	CLK4	DOWN
Phosphatidylinositol binding clathrin assembly protein	PICALM	DOWN
Phosphatidylinositol-4-phosphate 5-kinase, type I, α	PIP5K1A	DOWN
Phospholipase C, β 4	PLCB4	DOWN
Serine/threonine protein kinase D3	PRKD3	DOWN
Serine/threonine protein kinase Nek7	NEK7	DOWN
MAPK signaling pathway		
Fibroblast growth factor 2	FGF2	DOWN
cAMP-dependent protein kinase, β -catalytic subunit	PRKACB	DOWN
C-myc binding protein	MYCBP	DOWN
Serine/threonine protein kinase D3	PRKD3	DOWN
Serine/threonine protein kinase Nek7	NEK7	DOWN
Cell proliferation		
Fibroblast growth factor 2	FGF2	DOWN
Epiregulin	EPR	DOWN
BCL2-associated X protein	BAX	DOWN
Amphiregulin	AR	DOWN
Chemokine ligand 5	CXCL5	DOWN
CDC14 cell division cycle 14 homolog A	CDC14A	DOWN
Cell cycle		
Fibroblast growth factor 2	FGF2	DOWN
Cyclin E2	CCNE2	DOWN
Epiregulin	EPR	DOWN
BCL2-associated x protein	BAX	DOWN
Cyclin-dependent kinase-like 3	CDKL3	DOWN
CDC14 cell division cycle 14 homolog A	CDC14A	DOWN

^aFunctional groups of genes were analyzed on <http://david.abcc.ncifcrf.gov/>.

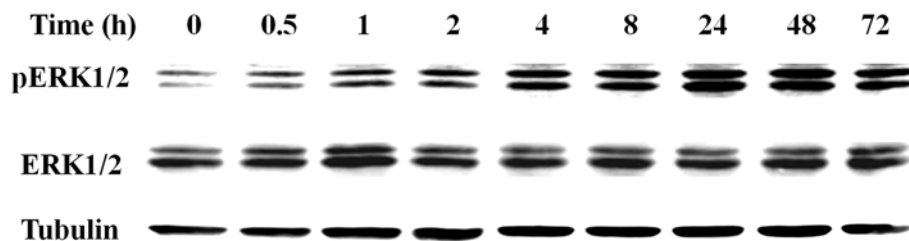


Figure 5. SNU449 cells were treated with 4.5 $\mu\text{mol/l}$ of Mibefradil for various time-points from 0 to 72 h. Cell lysate proteins were detected for pERK1/2, ERK1/2 and tubulin expression by Western blotting.

molecular mechanism of the T-type Ca^{2+} channels mediating cell proliferation process remains unclear. In current study, the effects of T-type Ca^{2+} channels blockade on HCC cell proliferation were examined. Blocking the functional T-type Ca^{2+} channel significantly decreased the proliferation of SNU449 cells. The result indicated that functional expression of T-type Ca^{2+} channels in some HCC cells probably have effect on the proliferation of these cells. However, it is yet uncertain that the observed T-type current was carried by single or multiple T-type $\alpha 1$ -subunits in SNU449 cells. In order to confirm whether $\alpha 1\text{G}$ subunit plays a predominant role in regulating SNU449 cell proliferation, RNAi approach was used to identify the role of $\alpha 1\text{G}$ subunit in cell proliferation. Silencing $\alpha 1\text{G}$ gene did not affect the proliferation of SNU449 cells. Furthermore, HepG2 cells were transfected with pcDNA3.1h $\alpha 1\text{G}$ plasmid. The result also demonstrated that the ectopic expression of $\alpha 1\text{G}$ gene did not stimulate cell proliferation. Therefore, we assumed that the observed functional T-type current in the SNU449 cells may be mainly controlled by $\alpha 1\text{H}$ subunit or multiple T-type $\alpha 1$ -subunits. Several studies have shown the overexpression or abnormal expression of $\alpha 1\text{H}$ T-type Ca^{2+} channel in some cancer cells, which suggested that $\alpha 1\text{H}$ subunit played a predominant role in regulating proliferation of these tumor cells (8,15).

Furthermore, in order to figure out how T-type Ca^{2+} channels affect cell proliferation, microarray analysis of SNU449 cells was performed based on gene biochip technology. The changes of more than 200 genes expression were documented. We mainly focused on those genes related to cell proliferation and cell cycle, and analyzed their possible roles in signal transduction pathways of tumor cells. Signal transduction in cancer cells often implicates the activation of receptor tyrosine kinase, and then triggers downstream multiple cytoplasmic kinases (16). Kikuchi *et al* had reported that Wnt signaling pathway was related to tumor formation and Ca^{2+} also played a certain role in this pathway (17).

Hepatocarcinogenesis is thought to implicate the multistep processes through the establishment of autocrine mechanisms for self-sustaining cellular growth (2,18). The epidermal growth factor receptor (EGFR) as a transmembrane tyrosine kinase is highly expressed in normal and transformed hepatocytes, and it can convey mitogenic and survival signals in transformed cells (19). EGFR can be bound and activated by some ligands, including EGF, transforming growth factor- α (TGF- α), heparin-binding EGF-like growth factor (HB-EGF), β -cellulin, epiregulin (EPR) and amphiregulin (AR). AR can

be as one key activator of the EGFR signaling pathway in the unrestrained growth and apoptosis resistance of HCC cells (20). It has also been reported that down-regulation of AR expression can induce the attenuation of basal EGFR and ERK1/2 phosphorylation, while basal EGFR and ERK1/2 phosphorylation are often activated in a significant proportion of human HCC (21). The ERK MAPK pathway as the extracellular signal-regulated kinase (ERK) pathway can be activated in various cells by diverse extracellular stimuli (22). Activation of the ERK MAPK pathway involves in the activation of a series of protein kinase. MAPK catalyzes the phosphorylation of ERK1/2. Finally they phosphorylate the various downstream substrates to regulate a variety of cellular responses such as cell proliferation, differentiation and apoptosis (23). EGF, AR or EPR can induce transient phosphorylation of ERK1/2, the phosphorylated ERK1/2 plays an essential role in transferring the signals to the nucleus and further regulates the transcription of some genes. Based on the microarray analysis, AR and EPR were down-regulated by Mibefradil. The down-regulated AR and EPR would possibly attenuate the activation of EGFR and downstream molecules of ERK MAPK and PI3K/AKT pathway, and ultimately affect gene transcription in the nucleus.

In addition, the increasing phosphorylated ERK1/2 was observed in the SNU449 cells with Mibefradil treatment. After the SNU449 cells were treated by 4.5 $\mu\text{mol/l}$ of Mibefradil for 4 h, the persistent increase of phosphorylated ERK1/2 lasted 72 h. The increased pERK1/2 may be associated with the growth inhibition of the SNU449 cells. Several studies have reported a strong correlation between persistent ERK1/2 phosphorylation and growth inhibition of cancer cells (24-26). According to MTT growth curve assay, the proliferation of SNU449 cells was reduced on the third day after Mibefradil treatment, which was probably related to the phosphorylation level of ERK1/2 in the SNU449 cells. Therefore, the growth arrest of SNU449 cells induced by Mibefradil may indicate two aspects. On one hand, Mibefradil down-regulated EGFR ligands and attenuated the EGFR signal pathway to reduce cell proliferation. On the another hand, Mibefradil induced the sustained increase in pERK1/2 to arrest cell growth.

The deregulation of the normal cell cycle may be one of essential causes in the progress of cancer. Cell cycle progression is mediated by cyclin-dependent kinases (CDKs). CDKs are activated via binding to specific cyclin proteins which are periodically synthesized in the cell cycle. Accumulation of the D-type cyclins in the G1 phase is necessary for



SPANDIDOS PUBLICATIONS progression. However, the cells can not complete cycle without cyclin E/CDK2 participation, because cyclin E/CDK2 can promote the phosphorylation of Rb and the release of E2F to contribute to the G1/S transition. Although cyclin D1 in SNU449 cells was not affected by Mibefradil, the cyclin E2 (CCNE2) and cyclin-dependent kinase-like 3 (CDKL3) were down-regulated in SNU449 cells by Mibefradil, which would likely lead to the change in cyclin activities, thus interrupt the cells through cell cycle and inhibit the cell proliferation. Some evidence has shown that alteration of cyclin E expression caused the development of many tumor types (27,28). Since normal expression and activity of cyclin E play a crucial role in cell cycle controls, any alteration in its expression possibly affects cell cycle progression.

In conclusion, the present study has shown that T-type Ca^{2+} channels probably play a certain role in regulating the proliferation of some HCC cells. Future studies will be required to examine the signaling pathways through T-type Ca^{2+} channels in modulating the cell cycle progression and the process of cell proliferation, especially the role of increasing pERK1/2 in activation of signal molecules and the transcription of some genes in the nucleus. Understanding the pathways of Ca^{2+} entry-dependent control of proliferation will help control disease progression and select better therapeutic strategies for cancer.

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