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Role of Caveolin-1 in Indomethacin-induced Death of Human Hepatoadenocarcinoma SK-Hepl Cells

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Caveolin-1 (CAV1) is an integral membrane protein that may function as a scaffold for plasma membrane proteins and acts as a tumor suppressor protein. One causative factor of chemotherapy-resistant cancers is P-plycoprotein (P-gp), the product of the multidrug resistance-1 gene (*MDR1*), which is localized in the caveolar structure. Currently, the interactive roles of CAV1 and MDR1 expression in the death of cancer cells remain controversial. In this study, we investigated the effects of indomethacin on the cell viability and the expression levels of MDR1 mRNA and protein in a CAV1-siRNA-mediated gene knockdown hepatoma cell line (SK-Hepl). Cell viability was significantly decreased in CAV1-siRNA-transfected cells compared with that of control-siRNA-transfected cells. Furthermore, the viability of cells pretreated with CAV1 siRNA was markedly decreased by treatment with indomethacin (400 μM for 24 h). However, the protein and mRNA levels of MDR1 were unchanged in CAV1-siRNA-transfected cells. These results suggest that CAV1 plays an important role as a major survival enzyme in cancer cells, and indomethacin can sensitively induce cell death under conditions of reduced CAV1 expression, independent of MDR1 expression.

Key Words: Caveolin-1, Multidrug resistance-1, siRNA, Indomethacin, SK-Hepl

INTRODUCTION

The caveolins (CAV1, −2, and −3) are integral membrane proteins of about 22~24 kDa that directly bind cholesterol and are the primary structural proteins of the caveolar membranes (Lavie et al, 1998; Okamoto et al, 1998; Liu et al, 2002). Of these isoforms, caveolin-1 (CAV1) is known to be a regulatory protein that interacts with the G-protein α subunit, H-Ras, the Src-family tyrosine kinases, protein kinase C isoforms, the EGF receptor, and eNOS, thus playing a negative regulatory role in signaling mechanisms (Li et al, 1995, 1996).

Recently, the overexpression of CAV1 has been reported in multidrug-resistant human cancer cells, including MCF-7 breast adenocarcinoma cells, HT-29 colon carcinoma cells, SKVLB1 ovarian carcinoma cells, and A549 lung carcinoma cells (Lavie et al, 1998; Yang et al, 1998; Bender et al, 2000; Belanger et al, 2004; Zhu et al, 2004). One of the causative factors of chemotherapy-resistant cancers, P-plycoprotein (P-gp), is the product of the multidrug resistance-1 gene (*MDR1*), and is expressed in the caveolar structure. It is an efflux pump protein for cytotoxic medicines, playing a survival role in cancer cells (Higgins, 1992; Scotto and Johnson, 2001).

However, the physiological effects of CAV1 on the activity or pattern of expression of MDR are controversial. In a number of human cancer cells that strongly express MDR1, CAV1 expression is increased or undetectable, suggesting that CAV1 expression levels are dependent or independent of those of MDR1 (Haber et al, 1995; Davidson et al, 2002). Furthermore, P-gp/MDR1 is localized in the caveolae and coimmunoprecipitates with CAV1, indicating that these proteins interact with one another (Demeule et al, 2000). Pang et al (2004) demonstrated the coordinated expression of CAV1 and MDR1 in normal and leukemic bone marrow, with a positive correlation in the expression levels of the two enzymes.

Hepatocellular carcinoma (HCC) is one of the most life-threatening cancers, and affects many of the world's populations (Farazi and DePinho, 2006). The control of MDR1 expression is a successful chemotherapeutic strategy for a variety of cancers, especially human HCC (Chen et al, 2006). CAV1 is highly expressed in the SK-Hepl human hepatoadenocarcinoma cell line, whereas only low levels of MDR1 are expressed.

Indomethacin is a substrate used as an anticancer drug. In this study, we hypothesized that if CAV1 interacts functionally or coordinately with MDR1 in indomethacin-

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ABBREVIATIONS: HCC, hepatocellular carcinoma; CAV1, caveolin-1; MDR1, multidrug resistance-1; P-gp, P-glycoprotein; si-RNA, small interfering RNA.

induced cell death, MDR1 expression might be altered by modulation of CAV1 expression, especially under CAV1-siRNA-induced gene knockdown.

METHODS

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), reduced- serum medium (Opti-MEM I), fetal bovine serum (FBS), streptomycin/penicillin, Lipofectamine™ 2000, and Trypan Blue stain were obtained from Invitrogen (Carlsbad, CA, USA). Indomethacin, dimethyl sulfoxide (DMSO), protease inhibitor mixture (P8340), and monoclonal antibody directed against β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caveolin-1 siRNA(h) (sc-29241), control siRNA-A (sc-37007), rabbit polyclonal anticaveolin-1 antibody, and mouse monoclonal antiMDR1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Oligonucleotides were obtained from Genotech Co. Ltd. (Daejeon, Korea).

Cell culture and treatment

SK-Hep1 cells, a human hepatoadenocarcinoma cell line, were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were grown in DMEM containing 10% FBS, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), in a humidified atmosphere of 5% CO_2 at 37°C. The cells were maintained in 100 mm culture dishes (TPP, Trasadingen, Switzerland), and seeded into six-well plates with 2×10^5 cells per well for the measurement of cell viability and gene expression. Indomethacin solubilized with dimethyl sulfoxide (DMSO) was stored at −20°C, and used at three different concentrations (100, 500, and 1,000 μM). The same volume of DMSO was used as the vehicle control. CAV1 expression levels were also partially knocked down using a CAV1-specific siRNA to control efflux activity, instead of adjusting MDR1 expression, because of the positive correlation between MDR1 and CAV1 expression described in other experiments (Lavie et al, 2001; Pang et al, 2004). Thus, SK-Hep1 cells were treated with 400 μM indomethacin in the presence or absence of CAV1 siRNA.

Transient transfection of CAV1 siRNA

As mentioned above, the cells were transfected with commercially available CAV1 siRNA using Lipofectamine™ 2000 (Invitrogen), following the manufacturer's protocol. One day before transfection, 2×10^5 cells per well in six-well plates were seeded into DMEM (2 ml) without antibiotics, and then incubated until 60~70% confluence for optimum transfection. After the cells had been rinsed with Opti-MEM I reduced-serum medium (Invitrogen), they were transfected with CAV1 siRNA-Lipofectamine™ 2000 or control siRNA-Lipofectamine™ 2000 complexes diluted to 30 nmol in the same medium for 4 h, after which growth medium containing 20% FBS was added. The transfected cells were maintained for 48 h.

Cell viability

To measure cell viability, the attached cells were washed with phosphate-buffered saline (PBS) and trypsinized. After the dissociated cells had been centrifuged at 2,000 rpm for 3 min, the supernatant was aspirated off, and the cells were gently resuspended with growth medium. The cells were stained with 0.4% Trypan Blue stain (Invitrogen) and counted using a Levy hemacytometer.

Reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated using TRI reagent (Molcular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RT-PCR was performed to semi-quantitatively measure the expression levels of CAV1 and MDR1 mRNAs relative to that of 18s rRNA as the internal control. Total RNA (1 μg) was reverse transcribed using an RNA PCR Kit (AMV version 2.1; TaKaRa, Shinga, Japan), according to the manufacture's protocol. The reaction mixture was incubated for 5 min at 30°C to anneal the primer to the RNA template, at 42°C for 30 min, and finally for 5 min at 99°C to inactivate the AMV reverse transcriptase. The cDNA generated was used for PCR amplification. Table 1 shows the sequences of the primer pairs used to amplify CAV1 and MDR1 mRNAs. The PCR mixture contained 200 mM dNTPs, 0.16 mM each of the sense and antisense primers, 1 unit of *Taq* DNA polymerase (TaKaRa), 3 μl of each cDNA, and sterile distilled water to a volume of 50 μl. PCR was carried out as follows: one cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and one final cycle of 5 min at 72°C. The amplified products were separated on 1.5% agarose gels in 1×TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.0) and stained with 1 mg/ml ethidium bromide.

Isolation of total cellular proteins and Western-blot analysis

Treated cells were washed with cold PBS and lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% SDS] containing 10% protease inhibitor cocktail (1 mM DTT, 2 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml

Table 1. Nucleotide sequences of primers used in RT-PCR analysis

Target	Primers	Sequences	Product size (bp)
Cav-1	Sense	5' −ACG TAG ACT CGG AGG GAC ATC TCT− 3'	508
	Antisense	5' −CTG CAA GTT GAT GCG GAC ATT GC− 3'	
MDR-1	Sense	5' −ATT CAA CTA TCC CAC CCG ACC G− 3'	324
	Antisense	5' −GCT GCC CTC ACG ATC TCT TCC T− 3'	
18S-rRNA	Sense	5' −TGC ATG TCT AAG TAC GCA CG− 3'	313
	Antisense	5' −TTG ATA GGG CAG ACG TTC GA− 3'	

pepstatin A, and 1 mM PMSF). Total protein concentrations were determined using a Pierce BCA Protein Assay kit (Pierce, Rockford, IL, USA). Equivalent amounts of total protein from each sample were resolved by electrophoresis on SDS-10% polyacrylamide gel. The gels were loaded in running buffer containing 24.9 mM Tris, 194 mM glycine, and 1.156 mM SDS at room temperature and run at 75 V. After the proteins were resolved, they were transferred to a PVDF membrane (Millipore, Miliford, MA, USA) by incubation overnight at 4°C and 30 V in transfer buffer containing 25 mM Tris, 192 mM glycine, and 10% methanol. The membranes were blocked with 5% nonfat dried milk in TBS-T buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% [v/v] Tween 20] at room temperature for 1 h. The membranes were then incubated at room temperature in fresh blocking buffer containing primary antibody directed against CAV1 (1 : 2,000 dilution), MDR1 (1 : 100 dilution), or β-actin (1 : 10,000 dilution). The duration of incubation at 4°C was 1 h to determine the levels of CAV1 and β-actin and overnight for MDR1. The membranes were then incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse IgG). The specific bands on the membrane were visualized on X-ray film (Kodak, Rochester, NY, USA) activated by chemiluminescence (ECL™ Western Blotting System; Pierce, Rockford, IL, USA). The intensity of each band was determined by densitometry using BIO-PROFILE software version 99.04 (Vilber Lourmat, France). Treatment of indomethacin decreased dose-dependently the viability of untreated cells (Fig. 1).

RESULTS

Effects of CAV1 siRNA and indomethacin on cell viability in CAV1-siRNA-treated cells

In the cells transiently transfected with CAV1-specific siRNA, cell viability was determined by direct cell counting. The effects of indomethacin on cell viability in cells pretreated with CAV1 siRNA were also compared. Treatment of indomethacin decreased dose-dependently the viability of untreated cells (Fig. 1). As shown in Fig. 2, CAV1 siRNA treatment for 24 h decreased cell viability by ~60% compared with the viability of the control-siRNA-treated cells, and indomethacin (400 μM, a toxic concentration) decreased the viability to ~75% of that of cells treated with only DMSO (the vehicle for indomethacin). Furthermore, cell viability was markedly decreased by treatment with indomethacin (400 μM for 24 h) in cells pretreated with CAV1 siRNA (Fig. 2). These results indicate that cell viability was significantly decreased by the synergistic effects of knockdown of CAV1 expression and the toxic activity of indomethacin.

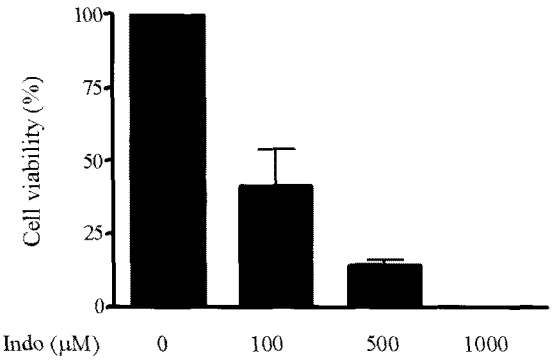

Fig. 1. Indomethacin-induced cell death. Cell viability was determined by cell counting after treatment with different concentrations of indomethacin (0, 100, 500, and 1,000 μM) for 24 h.

Effects of CAV1 siRNA and indomethacin on the expression of CAV1 and MDR1 mRNAs and proteins in CAV1-siRNA-treated cells

We investigated whether MDR1 expression levels are dependent or independent of the levels of CAV1, because other reports on normal or cancer cells have been controversial. In SK-Hep1 cells transiently transfected with control or CAV1 siRNA, the expression of CAV1 and MDR1 mRNA and protein levels were determined by semiquantitative RT-PCR and Western-blot analysis, respectively.

In cells treated with CAV1 siRNA, CAV1 mRNA expression was decreased to ~50% of the control levels (Fig. 3). However, the levels of MDR1 mRNA expression were unchanged, as shown in Fig. 3. Furthermore, indomethacin (400 μM) treatment decreased CAV1 mRNA expression by ~50% in the control cells and by ~70% in the CAV1-siRNA-treated cells, independently of the unchanged MDR1 levels (Fig. 3). The expression of these two enzymes at the protein level was consistent with that of the mRNA level, and CAV1 expression was independent of the expression of MDR1 (Fig. 4).

Effects of indomethacin on the expression of CAV1 and MDR1 mRNAs in untreated SK-Hep1 cells

Indomethacin treatment dose-dependently decreased CAV1 mRNA expression levels, whereas those of MDR1 mRNA were unchanged except at 1 mM indomethacin, at which MDR1 mRNA was reduced (Fig. 5). Cell death was severe (>95% cell death) at 1 mM concentration of indomethacin. However, unlike the CAV1 mRNA expression patterns,

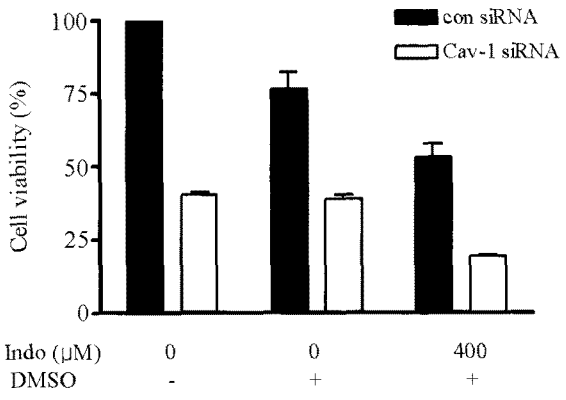

Fig. 2. Viability of CAV1-siRNA-transfected cells. Cell viability was determined by cell counting after treatment with indomethacin (400 μM) for 24 h. Cell viability was decreased significantly in CAV1-siRNA-transfected SK-Hep1 cells, and was markedly decreased by indomethacin treatment compared with that in each control.

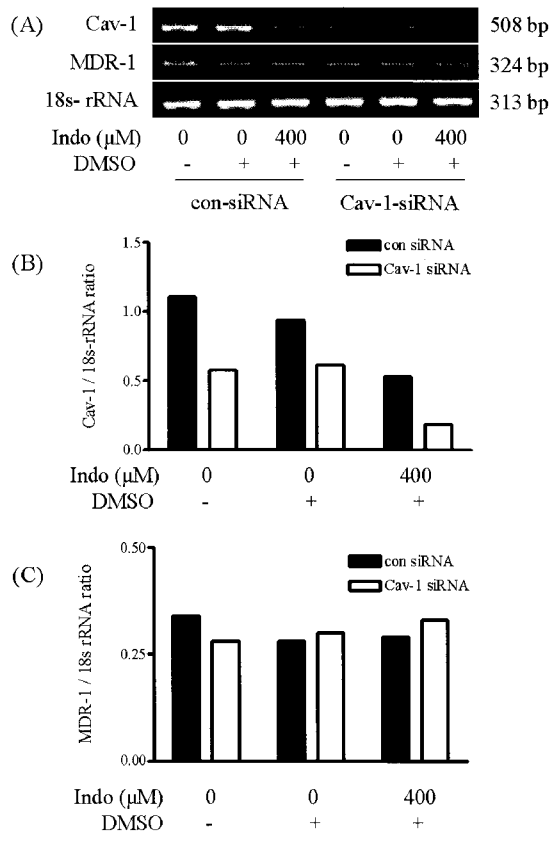

Fig. 3. Effects of indomethacin treatment on the expression of CAV1 and MDR1 mRNAs in CAV1-siRNA-transfected cells. Cells were first transfected for 24 h with control siRNA or CAV1 siRNA. To complete the knockdown of *CAV1* gene expression, a second transfection was performed for 24 h. Forty-eight hours after the first transfection, the cells were treated with indomethacin (400 μ M) for 24 h. DMSO was used as the vehicle. Expression levels of CAV1 (B) and MDR1 (C) mRNAs were determined by RT-PCR (A) and normalized against that for 18S rRNA.

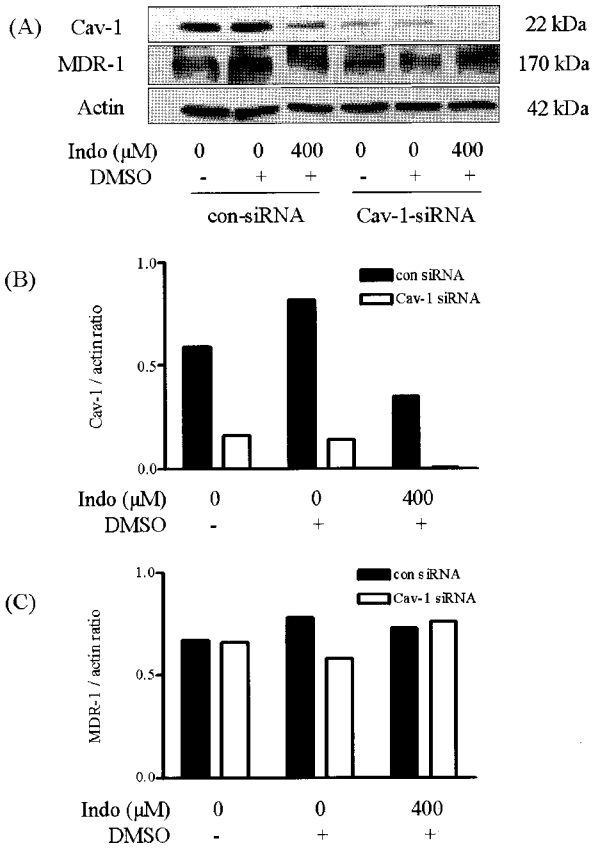

Fig. 4. Effects of indomethacin treatment on the expression of CAV1 and MDR1 proteins in CAV1-siRNA-transfected cells. Crude protein was extracted from the cells. DMSO was used as the vehicle. Expression levels of CAV1 (B) and MDR1 (C) proteins were determined by Western-blot analysis (A) and normalized against those for β-actin.

CAV1 protein expression did not change (Fig. 6). Thus, indomethacin treatment downregulates CAV1 mRNA only, with no change in CAV1 protein levels.

DISCUSSION

In this study, we examined the effects of indomethacin on the viability of SK-Hep1 human hepatoadenocarcinoma cells transiently transfected with control siRNA or CAV1-specific siRNA. We also examined whether the control of CAV1 expression was related to MDR1 expression.

Many patients with liver cancer suffer from resistance to chemotherapeutic drugs (Nerenstone et al, 1988). A membrane enzyme encoded by the *MDR1* gene acts as an efflux pump, protecting the cells from the cytotoxic effects of anticancer drugs (Higgins, 1992). Recently, several investigators have reported that MDR1 is associated with CAV1 in normal and cancer cells (Lavie et al, 1998; Demeule et al, 2000; Lavie et al, 2001; Pang et al, 2004; Cai et al, 2004; Zhu et al, 2004). However, Davidson (2002) suggested that MDR1 is not related to CAV1, particularly in ovarian cancers. Thus, the correlation between MDR1 and CAV1 enzyme activities remains controversial, because the related mechanisms underlying MDR1 and CAV1 expression are not fully understood.

We used the SK-Hep1 cancer cell line, one of the human hepatoadeno-carcinoma cell lines that overexpress CAV1 but only weakly express MDR1. In these cells, the control of CAV1 expression via transient transfection with CAV1-specific siRNA did not modulate MDR1 expression, at either the mRNA or protein level, as shown in Figs. 4 and 5. Thus, our results suggest that the functional activities of CAV1 and MDR1 are independent. Importantly, indomethacin, a known nonsteroidal anti-inflammatory drug, induces drug resistance in cancer cells via the upregulation of MDR1 expression (Rosenbaum et al, 2005). However, in our study, CAV1 was downregulated by indomethacin treatment, with no change in MDR1 expression, and indomethacin markedly induced cell death, especially in CAV1-siRNA-treated cells, relative to that in the controls.

Furthermore, the downregulation of CAV1 only, without indomethacin treatment, also resulted in ~50% cell death relative to that in the control siRNA-treated cells. These results suggest that CAV1 plays an important role in the survival of SK-Hep1 cells. As shown in Fig. 2, cell viability was significantly decreased by indomethacin treatment of CAV1-siRNA-transfected cells. Interestingly, Shatz and

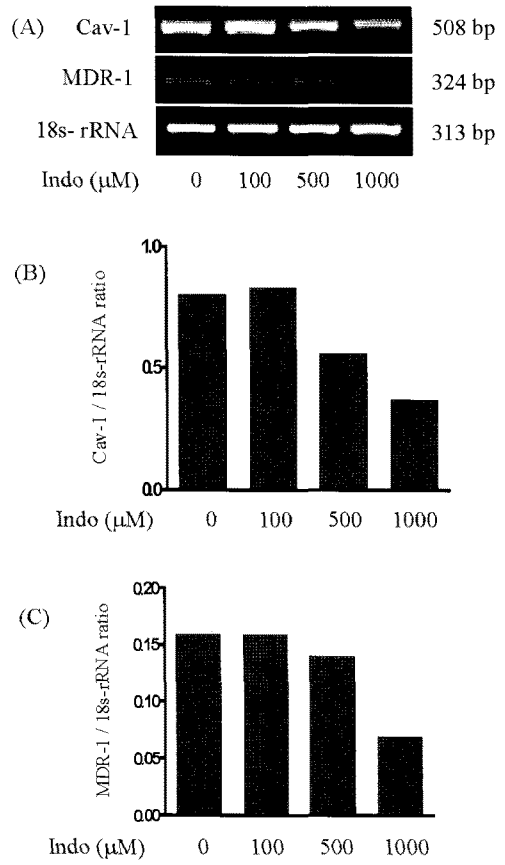

Fig. 5. Effects of indomethacin treatment on the expression of CAV1 and MDR1 mRNAs in SK-Hep1 cells. Cells were treated with different concentrations of indomethacin (0, 100, 500, and 1,000 μM) for 31 h. Expression levels of CAV1 (B) and MDR1 (C) mRNAs were determined by RT-PCR (A) and normalized against those for 18S rRNA.

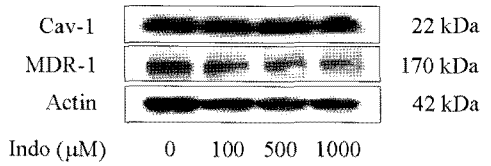

Fig. 6. Effects of indomethacin treatment on the expression of CAV1 and MDR1 proteins in SK-Hep1 cells. Crude protein was extracted from cells that had been treated with different concentrations of indomethacin (0, 100, 500, and 1,000 μM) for 31 h. Expression levels of CAV1 and MDR1 proteins were determined by Western-blot analysis and normalized against those for β-actin.

Liscovitch (2004) proposed that CAV1 has roles in both growth-inhibitory and survival-promoting actions, particularly as a regulator of prosurvival proteins together with MDR in cancer cells.

Therefore, our results suggest that CAV1 plays a major role in a survival mechanism, at least in the SK-Hep1 cancer cell line, independently of MDR1 expression. Further investigations are required to identify the molecular mechanism by which CAV1 regulates cell survival and modulates the expression of other genes in cancer cells.

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