Possible role of visfatin in hepatoma progression and the effects of branched-chain amino acids on visfatin-induced proliferation in human hepatoma cells

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Key words: visfatin, branched-chain amino acids, hepatocellular carcinoma.

Running title: BCAA suppresses visfatin-induced proliferation in HCC cells

Abbreviations: ANOVA, analysis of variance; BCAA, branched-chain amino acids; BMI, body mass index; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK-3\(\beta\), glycogen synthase kinase-3\(\beta\); HCC, hepatocellular carcinoma;
NAD, nicotinamide adenine dinucleotide; NAFLD, nonalcoholic fatty liver disease; MAPK, mitogen-activated protein kinase; MRI, magnetic resonance imaging; PBS, phosphate buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; SD, standard deviation; Stat3, signal transducer and activator of transcription 3.

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Abstract

Obesity and related metabolic abnormalities, including adipocytokine dysbalance, are risk factors for hepatocellular carcinoma (HCC). Visfatin, an adipocytokine that is highly expressed in visceral fat, is suggested to play a role in the progression of human malignancies. Branched-chain amino acids (BCAA) reduces the incidence of HCC in obese patients with liver cirrhosis and prevents obesity-related liver carcinogenesis in mice. In this study, we investigated the possible role of visfatin on HCC progression and the effects of BCAA on visfatin-induced proliferation of HCC cells. In patients with HCC, serum visfatin levels were significantly correlated with stage progression and tumor enlargement. Visfatin preferentially stimulated the proliferation of HepG2, Hep3B, and HuH7 human HCC cells compared with Hc normal hepatocytes. Visfatin phosphorylated ERK, Akt, and GSK-3β proteins in HepG2 cells. LY294002 (a PI3K inhibitor), PD98059 (a MEK1 inhibitor), CHIR99021 (a GSK-3β inhibitor), and BCAA significantly inhibited visfatin-induced proliferation in HepG2 cells. BCAA also inhibited phosphorylation of GSK-3β, increased cellular levels of p21<sup>CIP1</sup>, caused cell cycle arrest in G<sub>0</sub>-G<sub>1</sub>, and induced apoptosis in HCC cells in the presence of visfatin. These findings suggest that visfatin plays a critical role in the proliferation of HCC cells and may be associated with the progression of this malignancy. In addition, BCAA might inhibit obesity-related liver carcinogenesis by targeting and, possibly, by overcoming the stimulatory effects of visfatin.
Introduction

In addition to established risk factors such as hepatitis and alcohol consumption, obesity and its related metabolic abnormalities raise the risk of hepatocellular carcinoma (HCC) (1-4). Several pathophysiological mechanisms linking obesity and liver carcinogenesis have been demonstrated, including the emergence of insulin resistance and the subsequent inflammatory cascade (5). In obese individuals, increased adipose tissue leads to the expression of a variety of adipocytokines. Recently, the role of obesity-associated dysfunctional adipose tissue and subsequent adipocytokine dysbalance in carcinogenesis has attracted attention (6). Clinical trials have shown that adipocytokine disorders, including increased levels of leptin and decreased levels of adiponectin in the serum, are implicated in hepatocarcinogenesis (7, 8). Leptin induces proliferation and inhibits apoptosis in human HCC cells (9). These findings suggest that adipocytokine dysbalance may play an important role in the development and progression of HCC.

Visfatin/pre-B cell enhancing factor, which was originally isolated from peripheral lymphocytes, has been described as a secreted growth factor for early B cell proliferation (10). More recently, visfatin has also been characterized as an adipocytokine that is highly expressed in the visceral fat of humans and rodents. Increased levels of visfatin, which are positively correlated with the size of visceral fat deposits, are observed in various clinical conditions such as obesity and diabetes mellitus (11, 12). Abnormalities in serum levels of visfatin have also been reported in nonalcoholic fatty liver disease (NAFLD), which is a hepatic manifestation of metabolic syndrome (13). These results are somewhat conflicting, however, as both increased and decreased serum levels of this adipocytokine have been found in patients with NAFLD (14, 15).
Furthermore, previous studies have shown that visfatin may play a role in the development and progression of certain types of human malignancies (16). For instance, colorectal cancer, the development of which is associated with metabolic abnormalities (17), is accompanied by the overexpression of visfatin (18). Serum visfatin level is a good biomarker of colorectal malignant potential and stage progression (19). Visfatin stimulation increases cell proliferation in prostate and breast cancer cells (20, 21), whereas the use of visfatin inhibitor exerts an anti-tumor effect by inducing apoptosis (22). These findings suggest that visfatin is one of the key adipocytokines that links obesity and tumorigenesis and thus may be an effective target for the inhibition of obesity-related carcinogenesis. However, no detailed studies of the relationship between visfatin and HCC have yet been conducted.

Branched-chain amino acids (BCAA; leucine, isoleucine, and valine) is used in patients with liver cirrhosis to improve protein malnutrition (23). Recent clinical trials have demonstrated that oral supplementation with BCAA prevents progressive hepatic failure, improves event-free survival in patients with chronic liver diseases, and reduces the risk of HCC in these patients who are obese (BMI of 25 or higher) (4, 24). BCAA supplementation also prevents obesity-related carcinogenesis in both the liver and the colorectum of diabetic mice (25, 26). In the present study, we measured serum visfatin concentration in patients with HCC and examined whether it was correlated with stage progression and tumor enlargement. We also examined in detail the effects of visfatin on the acceleration of HCC cell proliferation, focusing on the activation of signaling pathways, and investigated whether BCAA suppresses visfatin-induced growth of HCC cells.
Materials and methods

Patients and measurement of serum visfatin concentration

Eighty-five primary HCC patients who underwent initial treatment at our hospital from January 2006 to December 2008 were enrolled in this study. Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (27). The greatest diameter of HCC was determined using dynamic CT or MRI. Fasting serum samples were collected at the time of diagnosis, and serum levels of visfatin were determined using ELISA (AdipoGen, San Diego, CA, USA). The study protocol was approved by the institutional review board for human research, and all patients gave written informed consents to enter the study.

Materials

Recombinant human visfatin was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). BCAA (total amino acid content, 12.28 mM), ΔBCAA (10.28 mM), and neutral amino acid media (12.28 mM) were obtained from Ajinomoto Pharmaceuticals Co. (Tokyo, Japan). ΔBCAA serves as basal medium and contains 17 amino acids except BCAA. The concentrations of amino acids in the medium are as follows (in mM): glycine, 0.40; alanine, 0.40; serine, 0.40; threonine, 0.80; cystine, 0.20; methionine, 0.20; glutamine, 4.00; asparagine, 0.40; glutamic acid, 0.40; aspartic acid, 0.40; phenylalanine, 0.40; tyrosine, 0.40; tryptophan, 0.08; lysine, 0.80; arginine, 0.40; histidine, 0.20; and proline, 0.40. BCAA medium was prepared by adding 2 mM BCAAs (0.952 mM leucine, 0.476 mM isoleucine, and 0.572 mM valine) to ΔBCAA medium. The composition of BCAA (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage used for the treatment of decompensated liver cirrhosis in Japan (4, 24). The neutral amino acid medium was prepared by adding 2 mM neutral amino
acids (0.667 mM each of alanine, serine, and glycine) to the ΔBCAA medium and served as an amino acid content-matched control for BCAA medium. LY294002 was purchased from Cell Signaling Technology (Beverly, MA, USA); PD98059, from Sigma (St. Louis, MO, USA); and CHIR99021, from Stemgent (San Diego, CA, USA).

**Cell lines and cultures**

HepG2, Hep3B, and HuH7 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and maintained in RPMI-1640 medium (Sigma) supplemented with 10% FCS. Hc human normal hepatocyte cell line was purchased from Cell Systems (Kirkland, WA, USA) and maintained in a CS-S complete medium (Cell Systems). The cell lines have been characterized by each source and any further authentication was not done in our laboratory. These cells were cultured in an incubator with humidified air with 5% CO₂ at 37°C.

**Cell proliferation assay**

Cell proliferation assays were performed using a cell proliferation kit (XTT, Roche, Mannheim, Germany) according to the manufacturer’s instructions. To examine the effects of visfatin on the proliferation of the HepG2, Hep3B, HuH7, and Hc cells, these cells were seeded on 96-well plates (1 x 10⁴ cells/well). After 16 hours of serum starvation, the cells were treated with the indicated concentrations (0 to 400 ng/mL) of exogenous visfatin for 48 hours in the absence of serum. To investigate the effect of LY294002, PD98059, CHIR99021, and BCAA, HepG2 cells were treated with these agents in the absence and presence of visfatin (100 or 400 ng/mL) for 48 hours in serum-free medium. All assays were performed in triplicate.
Protein extraction and Western blot analysis

Total cellular protein was extracted and equivalent amounts of protein were examined by Western blot analysis (28). The primary antibodies used to detect the respective protein bands have been described previously (28). An antibody to GAPDH was used as a loading control. The intensities of the blots were quantified with NIH Image software, version 1.62.

Cell cycle assays

Cell cycle assays were performed using a cell cycle detection kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer’s instructions. HepG2 cells were treated with BCAA for 48 hours in the absence and presence of 100 ng/mL visfatin. After the harvested cells were fixed and stained, they were analyzed for DNA histograms and cell cycle phase distribution using a FACScan flow cytometer (BD, Franklin Lakes, NJ, USA). The data were analyzed by using the CellQuest computer program (BD) as described previously (28).

Apoptosis assays

The Annexin V–binding capacity of treated cells was examined with flow cytometry using the Annexin V–FITC Apoptosis Detection Kit I (BD) to evaluate the induction of apoptosis. HepG2 cells were treated with BCAA for 48 hours in the absence and presence of 100 ng/mL visfatin. After the cultured cells were washed with cold PBS, they were incubated in Annexin V–FITC and propidium iodide (PI) for 15 minutes on ice. Stained cells were analyzed within 1 hour. Annexin V–FITC-positive and PI-negative cells were counted as apoptotic cells as described.
Statistical analysis

The data are expressed as mean ± SD. The statistical significance of the difference in mean values was assessed with one-way ANOVA, followed by Sheffe’s t-test. Values of $P < 0.05$ were considered significant.
Results

Association of serum visfatin concentration with HCC clinical stage and tumor size

We initially analyzed the possible association of serum visfatin concentration with the clinical stage and tumor size (greatest diameter) of HCC in 85 patients (54 men and 31 women, median age 73 years). The median serum visfatin concentration was 5.8 ng/mL (range 1.2-42.0). We found that the progression of clinical stage was correlated with serum visfatin concentration; the level of this adipocytokine was significantly increased in stage IV patients compared with levels in those with stage I and II disease ($P < 0.05$; Fig. 1A). In 85 patients, the mean Pearson product-moment correlation coefficient ($r$) and the $P$-value ($P$) of tumor size with serum visfatin concentration were 0.315 and 0.003, respectively (Fig. 1B). Moreover, similar results ($r = 0.326$ and $P = 0.01$) were obtained when patients with diabetes mellitus (HbA1c $\geq$ 6 %) and/or obesity were excluded (n = 53, Fig. 1C), indicating a positive correlation between HCC tumor size and serum visfatin levels regardless of complications with obesity and diabetes.

Effects of visfatin on cell proliferation and phosphorylation of ERK, Akt, and GSK-3β proteins in human HCC cells

We next examined whether visfatin stimulates the proliferation of HCC cells using XTT assay. When series of HCC cells (i.e., HepG2, Hep3B, and HuH7 cells) were treated with visfatin (25 to 400 ng/mL) for 48 hours, cell proliferation was significantly stimulated in a dose-dependent manner ($P < 0.05$; Figs. 2A, 2B, and 2C). In addition, treatment of HepG2 cells with 100 ng/mL of visfatin for 30 minutes caused a marked phosphorylation of ERK, Akt, and GSK-3β proteins (Fig. 2D), suggesting that visfatin might induce cell proliferation in HCC cells by activating PI3K/Akt and
Effects of PI3K, MEK1, and GSK-3β inhibitors on visfatin-induced proliferation of HepG2 cells

We next examined whether pharmacologic inhibitors of PI3K (LY294002), MEK1 (PD98059), and GSK-3β (CHIR99021) suppress visfatin-induced proliferation in HepG2 cells because the activation of PI3K/Akt and MAPK/ERK pathways might be involved in this proliferation (Fig. 2). As shown in Fig. 3, treatment with LY294002 (Fig. 3A), PD98059 (Fig. 3B), and CHIR99021 (Fig. 3C) significantly inhibited HepG2 cell proliferation both in the absence and presence of visfatin stimulation (100 and 400 ng/mL) ($P < 0.05$). These findings suggest that PI3K and MAPK pathways could be effective targets for the inhibition of visfatin-induced proliferation in HepG2 cells.

Effects of BCAA on visfatin-induced proliferation of HepG2 cells

BCAA is reported to suppress obesity-related liver carcinogenesis (4, 25). Therefore, we next examined whether BCAA inhibits visfatin-stimulated proliferation of HepG2 cells because this adipocytokine, which is increased in obese individuals (11, 12), might play a role in the progression of HCC (Fig. 1). As shown in Fig. 4A, the proliferation of HepG2 cells was significantly inhibited when the cells were treated in BCAA medium; meanwhile, this inhibition did not occur in neutral amino acid medium, which was served as an amino acid content-matched control for BCAA medium ($P < 0.05$). This finding possibly indicates that BCAA itself is specific in inhibiting the growth of HCC cells. In addition, a marked potentiation in the proliferative activity of HepG2 cells occurred after stimulation with 100 and 400 ng/mL visfatin, whereas BCAA treatment inhibited such proliferation in a dose-dependent manner regardless of
visfatin stimulation ($P < 0.05$). The inhibition of proliferation with 2 mM BCAA was greater (65% reduction) when the cells were cultured at higher concentration of visfatin (400 ng/mL) than that in the absence of the adipocytokine (41% reduction) (Fig. 4B). In contrast, cell proliferation was not induced when Hc normal hepatocytes were treated with similar concentrations of visfatin. BCAA also exerted no significant effect on the proliferation of Hc cells regardless of visfatin stimulation (Fig. 4C).

**Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells**

We next examined whether BCAA affected the phosphorylation of ERK, Akt, and GSK-3β proteins caused by visfatin in HepG2 cells. When the cells were stimulated by visfatin, the expression levels of p-GSK-3β protein were significantly decreased by BCAA treatment ($P < 0.05$; Fig. 5).

**Effect of BCAA on cell cycle progression, p21CIP1 expression, and apoptosis induction in HepG2 cells in the presence and absence of visfatin**

To determine whether the suppression of cell proliferation caused by BCAA (Figs. 4A and 4B) was associated with specific changes in cell cycle distribution, we performed cell cycle analysis using DNA flow cytometry. When HepG2 cells were stimulated by visfatin for 48 hours, the percentage of cells in G2/M–phase (38%) was increased compared to that of cells not stimulated by visfatin (18%). Furthermore, regardless of visfatin stimulation, BCAA treatment increased the percentage of cells in G0/G1–phase; the percentage of cells in this phase was increased from 59% to 71% in the unstimulated cells and from 48% to 70% in the stimulated cells (Fig. 6A). Expression levels of p21CIP1 protein, which suppresses tumors by promoting cell cycle
arrest (30), were also increased by BCAA treatment regardless of visfatin stimulation ($P < 0.05$; Fig. 6B). In addition, BCAA induced apoptosis in HepG2 cells because the percentage of Annexin V–positive cells was increased by the addition of BCAA in both the absence (2% to 27%) and the presence (2% to 10%) of visfatin stimulation (Fig. 6C).
Discussion

Obesity and related metabolic abnormalities are significant risk factors for the development of HCC (1-5). Among obesity-related metabolic disorders, adipocytokine dysbalance is considered to play a role in liver carcinogenesis (7-9); however, the detailed relationship remains unclear. The results of the present study provide the first evidence that higher levels of serum visfatin, which are frequently found in obese individuals (11, 12), are positively involved in stage progression and tumor enlargement in HCC. On the other hand, the serum levels of other adipocytokines, including leptin, adiponectin, and resistin, are not associated with the stage progression of this malignancy (data not shown). Furthermore, visfatin stimulation strongly induced proliferation in a series of human HCC cells but not in Hc normal human hepatocytes. These findings suggest that visfatin, which might act as a growth factor in HCC cells, is one of the key adipocytokines that links obesity and the progression of HCC. In addition, this study revealed that serum visfatin levels are significantly correlated with tumor enlargement of HCC in patients who are not obese and do not have diabetes mellitus. A recent report has demonstrated that visfatin is constitutively released from human HCC cells (31). This finding raises the possibility that visfatin is produced by HCC tissue itself, which might also explain the positive correlation between tumor size and serum visfatin levels observed in the present study. Therefore, our findings and the results of a previous report (31) together suggest that visfatin-dependent autocrine or paracrine loops contribute to abnormal proliferation in HCC cells.

The present study demonstrated that visfatin induced cell proliferation in HepG2 cells by activating PI3K and MAPK signaling pathways because visfatin stimulation significantly increased phosphorylation of Akt, ERK, and GSK-3β proteins.
in these cells. These findings are consistent with previous reports that visfatin regulates a variety of signaling pathways, including PI3K/Akt, MAPK/ERK, and Stat3 (20, 32, 33). Visfatin stimulation also increases cell proliferation and ERK activity in prostate cancer cells (20). Moreover, recent experimental studies have shown that the activation of PI3K/Akt, MAPK/ERK, and Stat3 pathways is significantly associated with the development of liver tumors in obese mice and that inhibiting the activation of these signaling pathways is critical to the prevention of obesity-related liver tumorigenesis (34, 35). These reports (34, 35), together with the present findings that specific inhibitors of PI3K, MEK1, and GSK-3β significantly suppress visfatin-induced proliferation in HCC cells suggest that visfatin and its related signaling pathways might be effective targets for inhibiting obesity-related liver carcinogenesis.

BCAA, which was originally developed to improve protein malnutrition in patients with liver cirrhosis (23), produces improvements in metabolic abnormalities, especially insulin resistance and glucose tolerance (36, 37). BCAA supplementation also reduces the weights of white adipose tissue and improves liver steatosis in mice fed a high-fat diet (38). In addition, long-term oral supplementation with BCAA is associated with a reduced frequency of HCC in obese individuals (4). In rodent models, BCAA prevents obesity-related liver and colorectal carcinogenesis, and their beneficial effects are involved in the amelioration of insulin resistance and reduction of serum leptin levels (25, 26, 39). In the present study, BCAA significantly inhibited the proliferation of HCC cells stimulated by visfatin without affecting that of normal hepatocytes. This mechanism is a new one of BCAA that might explain the suppressive effects of this agent on obesity-related tumorigenesis. Therefore, the evidences in the present and previous studies (4, 25, 39) strongly support the active administration of BCAA as an HCC chemopreventive agent in patients with liver
cirrhosis, especially obese patients who are at an increased risk for this malignancy. We are currently trying to gather evidence that BCAA prevents obesity-related liver carcinogenesis by targeting visfatin, in an ongoing animal study.

GSK-3β phosphorylation plays a critical role in cell survival, prevention of apoptosis, and progression of cell cycle in tumors (40). Therefore, the results of the present study suggest that BCAA might have inhibited visfatin-induced proliferation in HCC cells by, at least in part, inhibiting the phosphorylation of GSK-3β protein, which induces apoptosis and cell cycle arrest in the G0/G1–phase in HepG2 cells. These findings are significant when considering the possibility of BCAA as a chemopreventive agent for HCC because GSK-3β phosphorylation is closely associated with liver carcinogenesis (41). Phosphorylation of GSK-3β is also involved in the development of liver tumors in obese mice, and inhibition of this kinase effectively suppresses obesity-related liver tumorigenesis (35). Conversely, a recent study has shown that visfatin exerts antiapoptotic effects in HCC cells and this might be associated with the enzymatic synthesis of nicotinamide adenine dinucleotide (NAD)+ (15). FK866, a visfatin inhibitor, effectively inhibited cell growth and induced apoptosis in human HCC cells by reducing cellular levels of NAD+ (22). Further studies are required to clarify the effects of BCAA on the synthesis and regulation of NAD+ and their relevance to the chemopreventive characteristics of this agent.

In summary, our data explained, for the first time, the molecular mechanisms responsible for HCC cell proliferation induced by visfatin, establishing a direct association between obesity and HCC progression. Because the evaluation of obesity-related metabolic disorders such as insulin resistance and hyperleptinemia are useful for predicting the risk of recurrence in HCC (8, 42), we presume that, along with these metabolic abnormalities, measurement of serum visfatin levels might also have
the potential to become a valuable biomarker for HCC development and progression. The results of the present study also indicate that targeting visfatin and related signaling pathways might be a promising strategy for the prevention or treatment of HCC in obese patients with chronic liver disease. BCAA is potentially effective and critical candidate for this purpose because it can inhibit visfatin-mediated cell proliferation and activation of intracellular signaling pathways.
Figure legends

Figure 1. Correlation between serum visfatin concentrations and the clinical stage (A) and tumor size (B, C) of HCC. (A) and (B) The correlations were determined by analyzing 85 patients with primary HCC. (C) The correlation was determined by analyzing 53 HCC patients who are not obese and did not have diabetes mellitus. *P < 0.05.

Figure 2. Effects of visfatin on the cell proliferation and phosphorylation of ERK, Akt, and GSK-3β proteins in HCC cells. HepG2 (A), Hep3B (B), and HuH7 (C) cells were treated with the indicated concentration of visfatin for 48 hours in serum-free medium. Cell proliferation was evaluated using an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *P < 0.05. (D) HepG2 cells were treated with and without 100 ng/mL visfatin for 30 minutes and cell lysates were prepared. The cell lysates were then analyzed with a Western blot using respective antibodies. Equal protein loading was verified by the detection of GAPDH. Repeat Western blots yielded similar results.

Figure 3. Effects of inhibitors of PI3K, MEK1, and GSK-3β on visfatin-induced proliferation in HepG2 cells. HepG2 cells were treated with LY294002, a PI3K inhibitor (A), PD98059, a MEK1 inhibitor (B), or CHIR99021, a GSK-3β inhibitor (C), in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated using an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *P < 0.05.

Figure 4. Effects of BCAA on visfatin-induced cell proliferation in HepG2 cells. (A) HepG2 cells were treated in 2 mM BCAA or 2 mM neutral amino acid medium for 48 hours. Cell proliferation was evaluated using an XTT assay. HepG2 (B) and Hc (C) cells were treated with or without BCAA (0 mM, 0.5 mM, 1 mM, and 2 mM) in the
absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated using an XTT assay. Results were expressed as a percentage of the control value. Bars indicate SD values of triplicate assays. * \( P < 0.05. \)

**Figure 5.** Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3\( \beta \) proteins in HepG2 cells. HepG2 cells were treated with or without BCAA in the absence or presence of 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed by Western blotting using corresponding antibodies (left panels). The intensities of the blots were quantified using densitometry. Columns and lines indicate mean and SD (right panel). Repeat Western blots produced similar results. * \( P < 0.05. \)

**Figure 6.** Effect of BCAA on the progression of cell cycle, expression of p21\(^{\text{CIP1}} \), and induction of apoptosis in HepG2 cells in the presence and absence of visfatin. After treatment with and without BCAA in the presence and absence of 100 ng/mL visfatin for 48 hours, the cells were corrected and then used for cell cycle assay (A), Western blot analysis (B), and apoptosis assay (C). (A) The cells were stained with PI to analyze cell cycle progression. (B) Total proteins were extracted from the cells, and the cell extracts were analyzed with a Western blot using anti-p21\(^{\text{CIP1}} \) and GAPDH antibodies (upper panels). The intensities of the blots were quantitated using densitometry. Columns and lines indicate mean and SD (lower panel). * \( P < 0.05. \) (C) The cells were incubated with Annexin V–FITC to evaluate induction of apoptosis. Annexin V–FITC-positive and PI-negative cells were counted as apoptotic cells.
References


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Fig 1. (Ninomiya S.)

A

B

C

Clinical Stage

Tumor Size (cm)

VitD3 (ng/mL)

VitD3 (ng/mL)
Fig 2. (Ninomiya S.)

A

HepG2

0 25 50 100 150 200 250
Visfatin (ng/mL)

B

Hep3B

0 25 50 100 150 200 250
Visfatin (ng/mL)

C

HuH7

0 25 50 100 150 200 250
Visfatin (ng/mL)

D

Visfatin − +

Erk

pErk

Akt

pAkt

GSK-3β

pGSK-3β

GAPDH
Fig 5. (Ninomiya S.)

Western Blot Analysis

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ERK
pERK
Akt
pAkt
GSK-3β
pGSK-3β

Bar graphs show the ratio of phosphorylated proteins to total proteins for each condition.
Fig 6. (Ninomiya S.)

**A**  
**Cell Cycle Assay**

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**B**  
**Western Blot Analysis**

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**C**  
**Apoptosis Assay**

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