Branched Chain Amino Acid Suppressed Insulin-initiated Proliferation of Human Cancer Cells Through Induction of Autophagy

GIZACHEW YISMAW WUBETU[1], TOHRU UTSUNOMIYA[1], DAICHI ISHIKAWA[1], TETSUYA IKEMOTO[1], SHINICHIRO YAMADA[1], YUJI MORINE[1], SHUICHI IWAHASHI[1], YU SAITO[1], YUSUKE ARAKAWA[1], SATORU IMURA[1], HIDEKI ARIMOCHI[2] and MITSUO SHIMADA[1]

Departments of [1]Surgery and [2]Immunology and Parasitology, The University of Tokushima, Tokushima, Japan

Abstract. *Background: Branched chain amino acid (BCAA) dietary supplementation inhibits activation of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis in diabetic animal models. However, the in vitro effect of BCAA on human cancer cell lines under hyper-insulinemic conditions remains unclear. Materials and Methods: Colon (HCT-116) and hepatic (HepG2) tumor cells were treated with varying concentrations of BCAA with or without fluorouracil (5-FU). The effect of BCAA on insulin-initiated proliferation was determined. Gene and protein expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, respectively. Results: BCAA supplementation had no significant effect on cell proliferation and did not show significant synergistic or antagonistic effects with 5-FU. However, BCAA significantly decreased insulin-initiated proliferation of human colon and hepatic cancer cell lines in vitro. BCAA supplementation caused a marked decrease in activated IGF-IR expression and significantly enhanced both mRNA and protein expression of LC3-II and BECN1 (BECLIN-1). Conclusion: BCAA could be a useful chemopreventive modality for cancer in hyperinsulinemic conditions.*

The prevalence of metabolic syndromes is increasing in parallel with increasing cancer incidence worldwide. Former reports have indicated that obesity and its related metabolic abnormalities, especially diabetes mellitus, are associated with the development of certain types of human epithelial malignancies, including colorectal cancer (CRC) and hepatocellular carcinoma (HCC) (1-3). The risk of CRC is elevated in patients with metabolic syndromes (2,3). In addition, insulin resistance increases the risk of recurrence in HCC patients after curative treatment (4), indicating that the consequences of metabolic abnormalities in cancer are critical issues that need to be resolved.

The binding of insulin to cell surface receptors such as insulin receptor and insulin-like growth factor I receptor IGF-IR on tumor and pre-cancerous cells activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is responsible for cellular processes including proliferation and survival (4, 5). In animal models, exogenous insulin injection stimulated the growth of CRC precursors (6, 7). Elevated circulating insulin causes alterations in the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, which is involved in the development and progression of cancer (8, 9).

Therefore, clinical management to counteract insulin resistance and subsequent hyper-insulinemia should be taken to prevent the development of cancer. Alternatively, strategies to block the aberrant activation of insulin signaling of tumor cells will also be therapeutically relevant.

Dietary supplementation with branched chain amino acids (BCAA), comprising valine, leucine and isoleucine, has been suggested to improve protein malnutrition in patients with liver cirrhosis (10) and is useful for preventing progressive hepatic failure and improving event-free survival in patients with chronic liver diseases through improving insulin resistance (11, 12). Shimizu *et al.* (13) reported that BCAA dietary supplementation improved insulin resistance and inhibited the activation of the IGF/IGF-IR axis, thereby, preventing the development of colonic pre-malignancies in an obesity-related colon cancer model. However, the *in vitro* effect of BCAA in human cancer cell lines needs to be clarified.

Autophagy is considered to be a major approach of killing apoptosis-resistant tumor cells (14). The microtubule-associated protein light chain (LC3-I) is conjugated to

Correspondence to: Mitsuo Shimada, MD, Professor and Chairman, Department of Surgery, The University of Tokushima 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Tel: +81 886337137, Fax: +81 88631 9698, e-mail: mitsuo.shimada@tokushima-u.ac.jp

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phosphatidylamine to form LC3-phosphatidylamine, termed LC3-II. A decrease in LC3-I and increased LC3-II levels are markers that reflect the activation of autophagy. The insulin/IGF-I–PI3K–AKT–mTOR pathway has been revealed to regulate cell autophagy through the insulin receptor (15). In addition, it has been demonstrated that activation of insulin/IGF signaling can suppress the autophagic lysosomal pathway (16).

The prevention of cancer by targeting the activation of the IGF/IGF-IR axis might be a promising strategy for obese people who are at increased risk of cancer. Therefore, investigation of novel candidate chemopreventive agents is critical to identify potential treatment solutions for populations in which excessive body weight has been found to be associated with the risk of various types of human epithelial malignancies (17, 18).

Herein, we investigated the *in vitro* effect of BCAA alone or in combination with fluorouracil (5-FU) in human colon and hepatic cancer cell lines and explored the possible mechanisms by which BCAA inhibits insulin-initiated cancer cell proliferation.

Materials and Methods

Antibodies and reagents. BCAA mixture (Leu/Ile/Val=2:1:1.2) was obtained from Ajinomoto Pharmaceuticals, Tokyo, Japan, and was dissolved in medium as *per* the recommendation from the company. Rabbit monoclonal anti-phospho-IGF-IR (Tyr980), rabbit polyclonal anti-LC3I/II, rabbit polyclonal anti-BECLIN-1and horseradish-peroxidase (HRP)-linked anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-IGF-IR antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-GAPDH monoclonal antibody was purchased from Abcam (ab9484; Cambridge, MA, USA) and insulin was obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell culture. Human colorectal cancer HCT-116 and HCC HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in McCoy's 5A (GIBCO, life technology) (HCT-116) and RPMI(Wako Pure Chemical Industries, Ltd) (HepG2) medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in an atmosphere with 5% CO_2 at 37˚C. For proliferation assays, cells were pre-cultured in the BCAA mixture supplemented with the appropriate medium for 1 h before treatment with insulin. For western blotting and gene expression analysis, cells were replenished with medium containing BCAA at the indicated concentrations for 30 min before insulin stimulation.

Cell proliferation assays. Cell proliferation assays were carried out using the WST-8 assay with a Cell Counting Kit-8 (CCk-8), (Dojindo Laboratories, Kumamoto, Japan). Briefly, 5×10^3 cells/well were seeded in 96-well plates, incubated for 24 h and then medium containing BCAA at various concentrations was added and the incubation continued for up to 72 h. After 24, 48 and 72 h incubation, 10 μL of CCK-8 reagent provided with the kit was added to each well and the plates were incubated at 37˚C in an atmosphere of 5% CO_2 for 4 h. Finally, the optical density was measured with a microplate reader (Multiskan JX; MTX Lab Systems, Vienna, VA, USA) at a wavelength of 450 nm.

Quantitative real time polymerase chain reaction (qRT-PCR). Total RNA was extracted from cells after treatment with insulin or BCAA-insulin combined for 48 h using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2.5 μg total RNA by reverse transcription using the Super Script RT kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. qRT-PCR was performed using the Applied Biosystems 7500 real time PCR system, TaqMan Gene Expression Assays on demand and TaqMan Universal Master Mix (gene specific TaqMan probes on a StepOne Plus; Applied Biosystems, Foster City, California, USA). Human *IGF-IR* (Hs00609566_m1), *BECN1* (Hs00186838_m1), *MAPLC3* (Hs01076567_g1), *BAX* (Hs00180269_m1) and *BCL2* TaqMan primers were used. GAPDH was used as an internal control for normalization. Expression levels of all genes were calculated as a ratio to *GAPDH*. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1 (Applied Biosystems).

Measurement of cell apoptosis. HCT-116 cells were grown in a monolayer before treatment with the indicated concentrations of BCAA with insulin or insulin alone for 48 h. Cells were washed twice with cold PBS and then re-suspended in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. Transfer of 100 μl of solution (1×10^5 cells) to a 5 ml culture tube was performed and 5 μl of FITC Annexin V (BD Pharmingen, San Diego, CA, USA) was then added. The cells were gently vortexed and incubated for 15 min at room temperature (25˚C) in the dark. Then, 400 μl of 1X Binding Buffer was added to each tube. Analysis was performed with a flow cytometer (Beckman Coulter, Brea CA, USA) to identify the subpopulations of the apoptotic cells within 1 h.

Western blot analysis. Cellular protein was extracted according to standard protocols. Briefly, cells were lysed in ice-cold RIPA buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Scientific Inc.] containing a protease inhibitor cocktail (10 μl/ml, sigma) and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA, USA). For immunoblotting, 30-50 μg protein were resolved over 7.5-12% polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking in 5% non-fat dry milk in TBS, the membranes were incubated with primary antibodies at 1:500 dilution in TBS overnight at 4˚C, washed three times with TBS-Tween 20 and then incubated with secondary antibodies conjugated with horseradish peroxidase at 1:2000 dilution in TBS for 2 h at room temperature. Membranes were washed again three times in TBS-Tween 20 at room temperature. After final treatment with enhanced chemiluminescence assay (ECL) reagents (GE Healthcare, Life Science,UK), samples were exposed to x-ray film to detect the protein bands.

Statistical analysis. Data are expressed as the mean±standard error of the mean (SEM) from a minimum of three separate experiments. The differences between means in two groups were analyzed by the Student's *t*-test. One way analysis of variance (ANOVA) with a Bonferroni multiple comparison *post-hoc* test was used when two

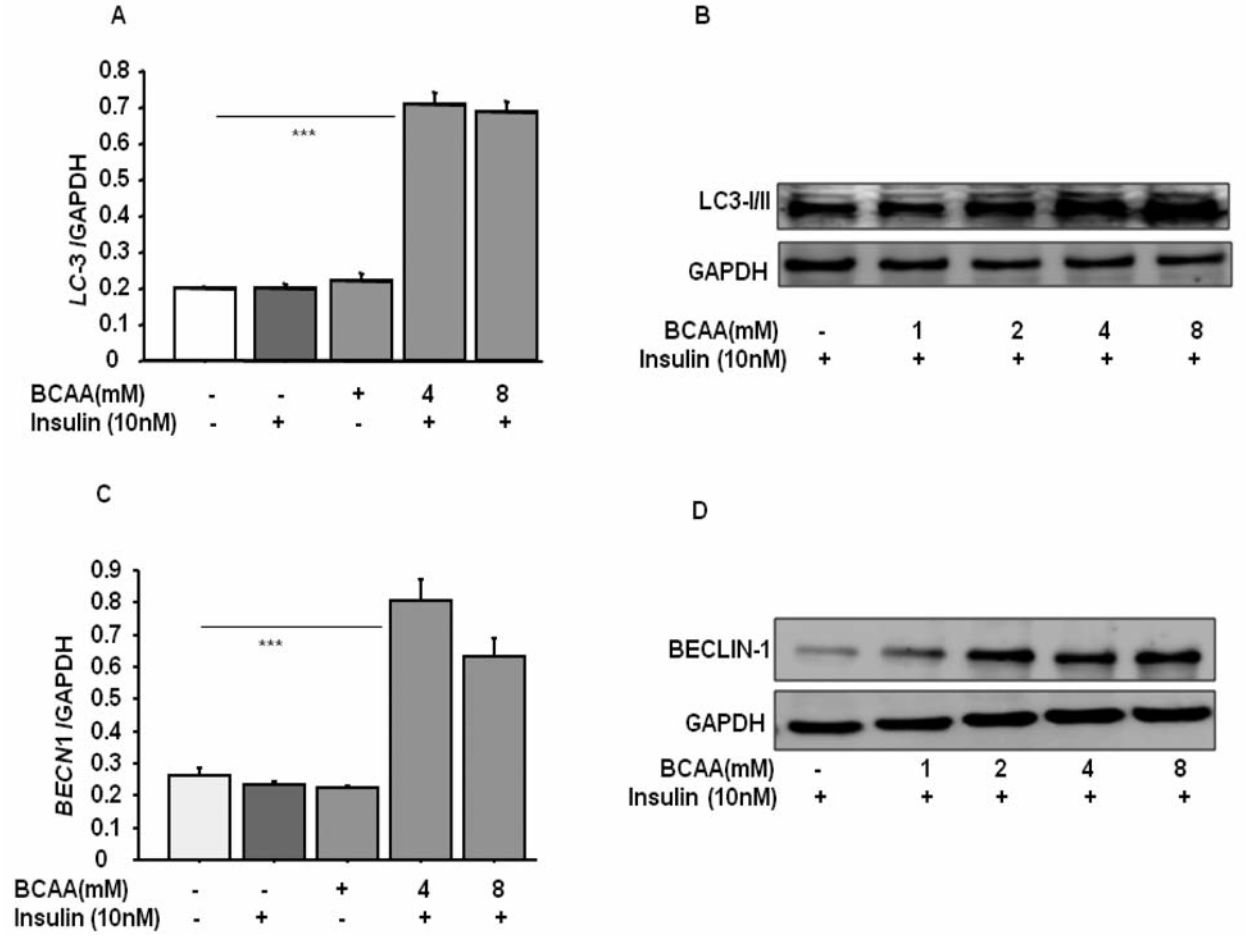

Figure 1. *Effect of BCAA supplementation on the expression of autophagic indicators. (A) qRT-PCR analysis of mRNA expression of LC-3; (B), western blot analysis of protein expression of LC-3; (C), qRT-PCR analysis of mRNA expression BECN1; (D), western blot analysis of protein expression of Beclin-1 (insulin or BCAA-alone vs. insulin + BCAA).*

or more groups were compared. Statistical analyses were performed using the SPSS for Windows version 17.0 (SPSS Inc, Chicago, IL, USA). Statistical significance was determined at $p<0.05$

Results

BCAA supplementation decreased insulin-induced proliferation of human colon and hepatic tumor cells in vitro. As shown in Figure 1, we first checked the effect of BCAA-alone or in combination with 5-FU on colon (HCT-116) and hepatic (HepG2) cancer cell lines. BCAA supplementation had no significant time- or dose-dependent effects on cell proliferation (Figure 1A, B), nor did it show significant synergistic or antagonistic effects with 5-FU (Figure 1C, D).

We next investigated the *in vitro* effect of BCAA on insulin-induced cell proliferation. Treatment with insulin for 48 h significantly increased proliferation of HCT-116 cells but not HepG2 cells. BCAA supplementation at more than 2 mM significantly decreased insulin-induced cell proliferation in HCT-116 cells, whereas BCAA at more than 4 mM significantly suppressed insulin-initiated proliferation of HepG2 cells in a dose-dependent manner. Differences in the sensitivity to insulin were observed among the two cell lines. Because HCT-116 showed a relatively high susceptibility to insulin-induced cell proliferation, HCT-116 cells were used for the following in-depth analyses.

Suppression of phosphorylation of IGF-IR by BCAA in HCT-116 cells. To understand the mechanisms underlying the suppressive effect of BCAA on insulin-induced cell proliferation, we determined the mRNA expression level of *IGF-IR* in cells treated with insulin-alone compared to insulin and BCAA combined for 48 h (Figure 2A). Insulin enhanced the mRNA expression levels of *IGF-IR*, which was

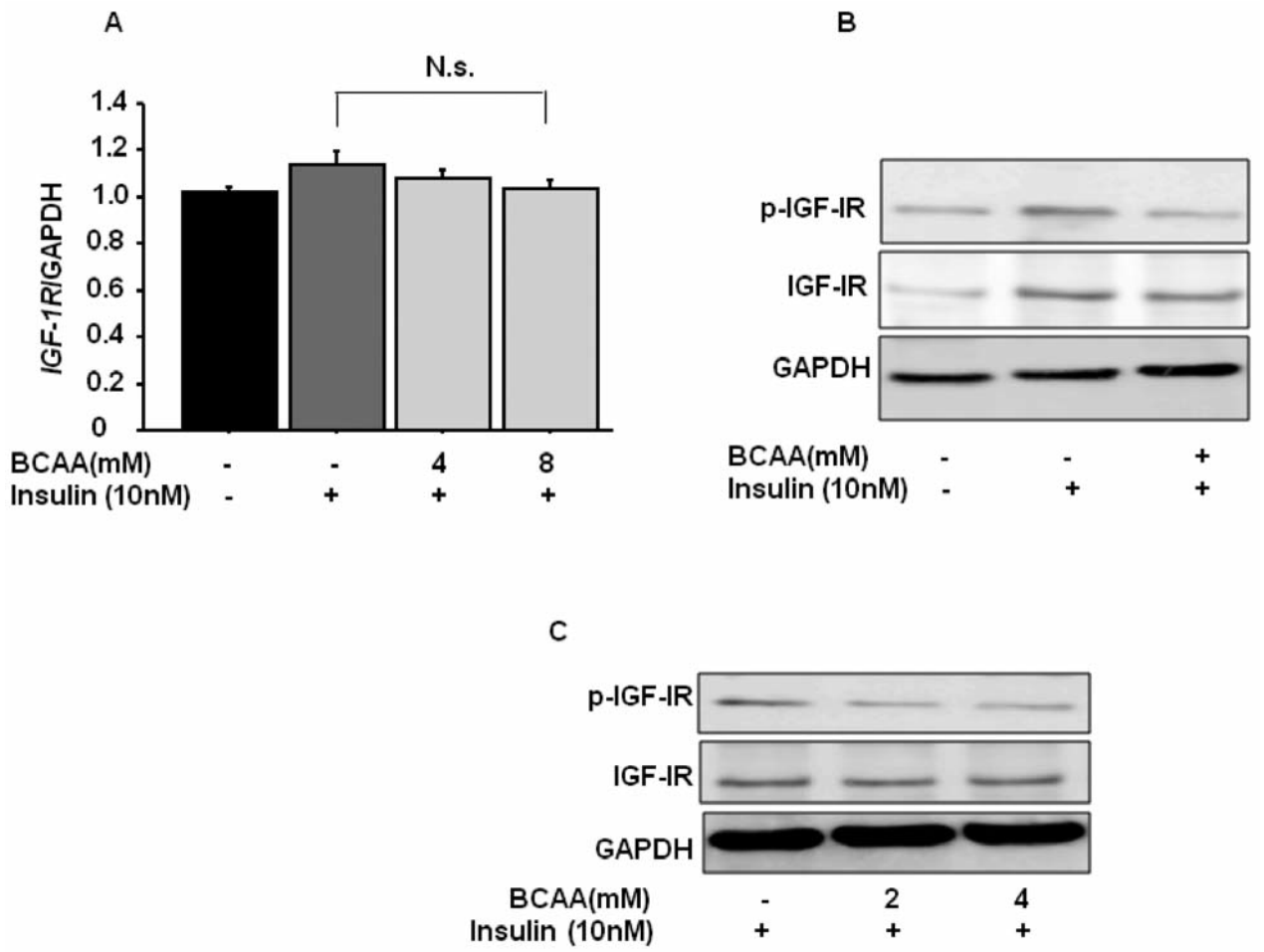

Figure 2. *Effect of BCAA on mRNA and protein expression of IGF-IR in HCT-116 cells. (A), qRT-PCR analysis of mRNA expression; (B) and (C), western bolt analysis of p-IGF-IR and total IGF-IR (insulin alone vs. insulin + BCAA).*

down-regulated by BCAA supplementation, though not significantly. We then speculated that BCAA could inhibit the activation (phosphorylation) of IGF-IR and therefore checked the effects of BCAA on the total and phosphorylated forms of IGF-IR by Western blot analysis using specific antibodies. Phosphorylation of IGF-IR was enhanced by 12 h insulin treatment, whereas p-IGF-IR was reduced by BCAA supplementation, but no notable effect was observed in the expression of total IGF-IR (Figure 2B). Furthermore, BCAA reduced the level of insulin-initiated activation of IGF-IR in a concentration-dependent manner and the suppressive effect of BCAA on p-IGF-IR was more effective at 2 mM (Figure 2C).

BCAA enhanced autophagy in HCT-116 cells. The IGF/IGF-IR axis controls the expression and function of many proteins that are involved in the regulation of cell survival including autophagy and apoptosis. To shed light on the autophagic profiles of BCAA, we measured the expression of LC-3 and *BECN-1* at the gene level as well as LC-3 and BECLIN-1 at the protein level using qRT-PCR and western blot analysis, respectively, to show the effect of BCAA on autophagy in cells treated with insulin-alone or insulin and BCAA in combination (Figure 3A-D). We observed BCAA dose-dependently up-regulated LC3-II and *BECN-1*(BECLIN-1) expression at both the gene and protein level in cells stimulated with insulin.

BCAA supplementation did not induce apoptosis of HCT-116 cells. Apoptosis assays were performed in cells treated with insulin with or without BCAA for 48 h. No significant differences in apoptosis were observed between the groups in triplicate experiments (Figure 4A-C). In addition, qRT-PCR mRNA analysis of apoptotic markers (*BAX* and *BCL2*), further confirmed the lack of significant difference between the groups (Figure 4E-F).

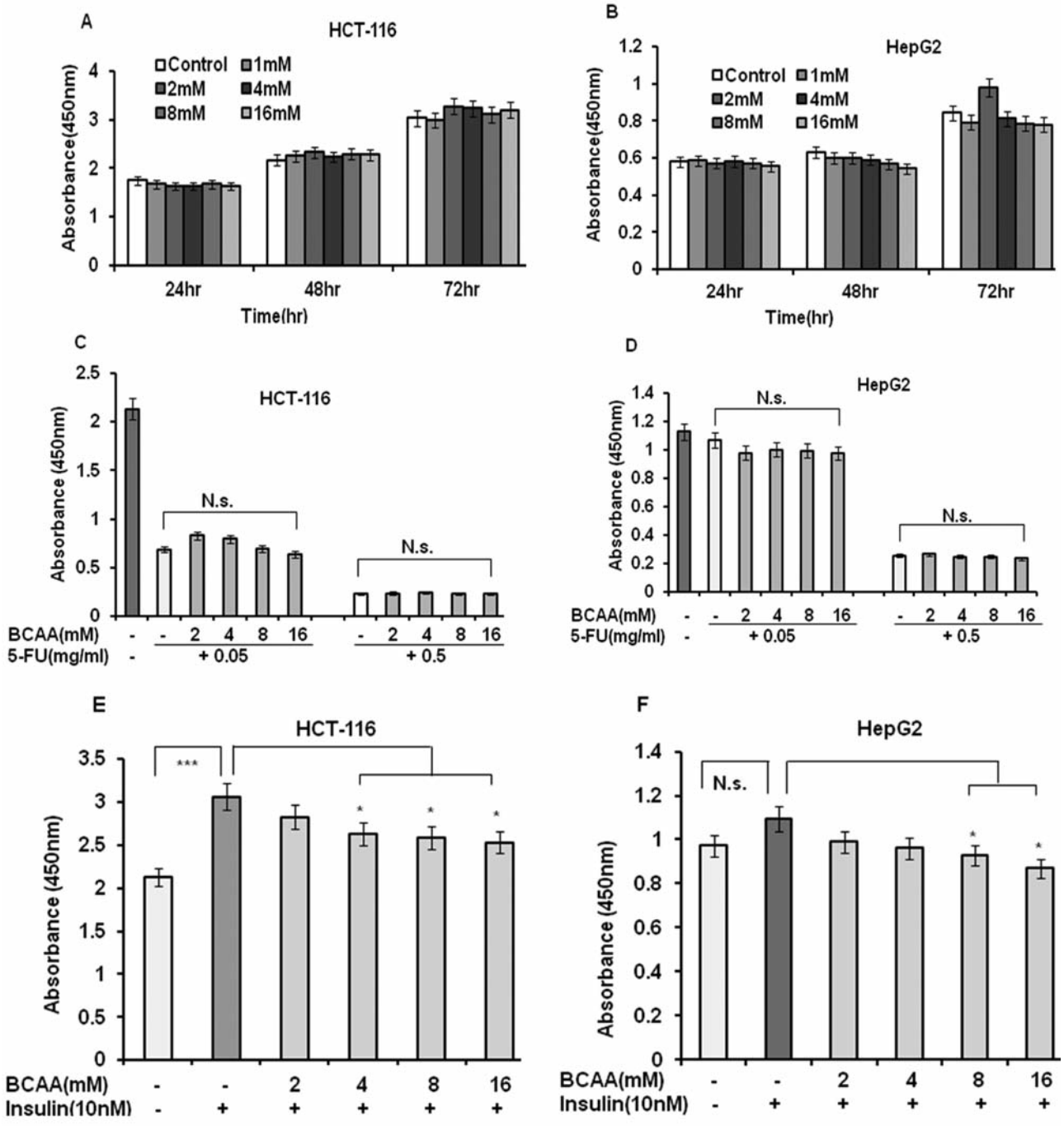

Figure 3. *Effect of BCAA on human colon (HCT-116) and hepatic (HepG2) tumor cell proliferation. (A) and (B), timecourse effect of BCAA on tumor cell proliferation (control vs. BCAA-alone); (C) and (D), combined effect of varying concentrations of BCAA and 5-FU on tumor cell proliferation (5-FU alone vs. 5-FU + BCAA); (E) and (F), effect of BCAA on insulin-induced proliferation of tumor cells (control vs. insulin alone, and insulin alone vs. insulin + BCAA, respectively). In all cases, cell viabilities were measured by WST-8 assay. Values are expressed as absorbance at 450 nm and the results are expressed as mean±SEM (n=5). *p<0.05, ***p<0.001, N.s. not significant.*

Discussion

The emergence of insulin resistance and alterations in the IGF-I/IGF-IR axis has been linked with colorectal and liver carcinogenesis (1, 2, 17). This suggests that regulation of these disorders *via* nutritional or pharmaceutical intervention might be an effective and promising strategy to inhibit insulin-related carcinogenesis.

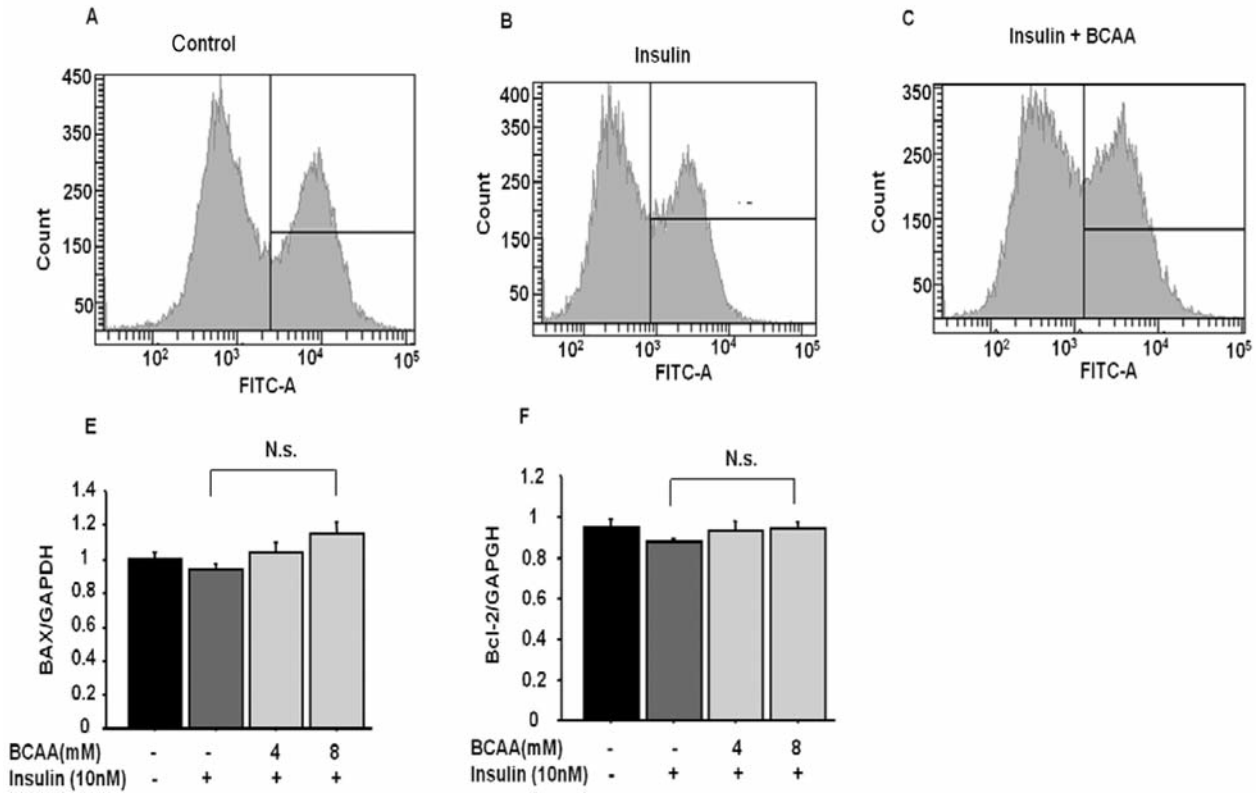

Figure 4. *Effect of BCAA on apoptosis and mRNA expression of apoptotic markers in HCT-116 cells. (A-C) Flow cytometry analysis of apoptotic cells; (E) and (F), qRT-PCR analysis of mRNA expression of apoptotic markers, BAX and BCL2 (insulin-alone vs. insulin + BCAA).*

In recent years, BCAA dietary supplementation has been shown to improve protein malnutrition (10) and reduce insulin resistance, progressive hepatic failure and the risk of HCC in obese cirrhotic patients (11, 12). In obese and diabetic mouse and rat models, BCAA prevents obesity-related liver and colorectal carcinogenesis through the amelioration of insulin resistance (13, 19, 20).

BCAAs, especially leucine, activate mTORC1 (5, 10) and thus BCAA supplemental therapy could potentially promote tumor progression. However, clinical and experimental studies have demonstrated that BCAA in fact decreases tumor incidence by maintaining the nitrogen balance and hence improving prognosis and survival (12, 21). In the present study, BCAA alone exerted no significant effect on the viability of human colon and hepatic tumor cells *in vitro*, which was in agreement with a previous *in vitro* study using rat hepatic tumor cells (5). In addition, BCAA supplementation did not alter the effect of 5-FU.

Insulin stimulates the proliferation of tumor cells and promotes tumor growth in animal models; however, BCAA supplementation significantly inhibited tumor cell proliferation (6, 7). In chemically-induced hepatocarcinogenesis model animals, dietary supplementation with BCAA also prevented obesity-related carcinogenesis (13, 19). In a recent *in vitro* study using rat hepatic tumor cells, BCAA suppressed insulin-induced cell proliferation (5), which is in agreement with the present findings using human colon and hepatic cancer cell lines. Altogether, these results support the administration of BCAA as a tumor-preventive agent for those who are at high risk of insulin-related malignancies.

Insulin activates mitogen-activated protein kinase (MAPK). It also cross-reacts with IGF-IR and further activates the RAF/MAPK kinase/MAPK cascade (4, 23). The possible mechanisms for the inhibitory effects of BCAA on carcinogenesis may include BCAA activation of the insulin signaling cascade to promote glucose uptake through activation of PI3K and subsequent translocation of glucose transporters (GLUT1 and GLUT4) which are integral membrane proteins, binding of glucose to one site provokes a conformational change associated with transport, and releases glucose to the other side of the membrane in the skeletal muscle (4, 8), followed by reduction of serum insulin levels and inhibition of the IGF/IGF-1R axis by suppressing the expression of IGF-1, IGF-2 and IGF-IR (19).

Moreover, in mice fed a high-fat diet, BCAA supplementation ameliorated insulin resistance by improving adipocytokine imbalance, inhibiting lipid accumulation in the liver, and increasing the hepatic levels of peroxisome proliferator-activated receptor-α (6, 13). Several clinical trials have also reported that oral BCAA supplementation improves glucose tolerance and insulin resistance in patients with chronic liver disease (4, 11).

A study using HepG2 cells demonstrated that supplementation with BCAA decreased insulin resistance-induced expression of endothelial growth factor (EGF) and subsequently suppressed tumor angiogenesis (22). Yoshiji *et al*. (20) also reported that the chemopreventive effect exerted by BCAA supplementation against HCC in obese and diabetic rats was associated with suppression of vascular endothelial growth factor (VEGF) expression and hepatic neovascularization. In a previous *in vitro* study in rat hepatic tumor cells, BCAA suppressed insulin-induced overactivation of PI3K/AKT and exhibited growth inhibitory effects by inducing apoptosis (5). In the present study, BCAA supplementation inhibited insulin-initiated proliferation in human cancer cell lines, which could occur partly through inhibition of activation of IGF-IR followed by autophagy induction. Thus, the diverse effects of BCAAs on insulin signaling may suppress carcinogenesis and could potentially affect the progression of metabolic-related malignancies.

Insulin receptor substrate (IRS-1) is an adaptor of IGF-IR, which plays an important role in transmitting signals from the IGF-IR to intracellular pathways, including the PI3K/AKT/mTOR pathway. Tyrosine phosphorylation of IGF-IR induces the cytoplasmic binding of IRS-1 to IGF-IR and phosphorylation of multiple tyrosine residues in IRS-1. This enables IRS-1 to activate the PI3K pathway (4, 5). Abnormal activation of the IGF/IGF-IR axis plays a critical role in obesity-related cancer development and is involved in cancer cell survival, prevention of apoptosis and cell-cycle progression (23). In line with our findings, previous *in vivo* studies have reported that BCAA supplementation inhibited activation of IGF-IR (13, 19, 20). Our observations described herein comprise the first report showing that BCAA decreased the proliferation of insulin-induced human cancer cell lines and p-IGF-IR *in vitro*. BCAA might inhibit insulin-induced proliferation of human cancer cell lines, at least in part, by inhibiting the activation of IGF-IR.

The growth factor signaling involved in the insulin/IGF-I-PI3K-Akt-mTOR pathway has been revealed to regulate cell autophagy through the insulin receptor (15). In addition, it has been revealed that activation of IGF-IR signaling can suppress the autophagic lysosomal pathway (16, 24). An inhibitory role for IGF-IR in autophagy has been observed in different cell types, including human osteocarcinoma cells (25).

The role of autophagy in cancer is paradoxical. However, down-regulated expression of autophagy-related genes are associated with colorectal tumorigenesis (26, 27), while induction of autophagy contributes to proliferative arrest of human colorectal cancer cells (28, 29). Several lines of studies suggest cytotoxic agents, including chemotherapeutic agents, induce cancer cell autophagy (7, 16). In the present study, we observed BCAA enhanced *LC3-II* and *BECN1* expression in human cancer cells, indicating BCAA may also induce cancer cell autophagy.

With regards to colorectal cancer, the majority of data indicated the role of autophagy in the delay of apoptosis, but the mechanism underling this antagonistic relationship is unclear. One theory proposed that autophagic degradation of damaged mitochondria prevents the release of pro-apoptotic factors, such as cytochrome *c* (30).

In the present study, increased expression of autophagic markers and inhibition of p-IGF-IR in the presence of BCAA suggests that BCAA-IGF-IR signaling may be involved in the regulation of autophagy in HCT-116 cells. However, further studies using autophagy inhibitors are required to strengthen the present findings.

In summary, among human cancers CRC and HCC are the most representative malignancies affected by metabolic abnormalities. In the present study, we demonstrate for the first time that BCAA may suppress insulin-induced proliferation of human colorectal tumor cells by inducing autophagy. Our findings suggest that the inhibition of IGF/IGF-IR activation by BCAA could play an important role in suppressing insulin-related carcinogenesis. Thus, active intervention using BCAA might be a promising approach for the chemoprevention of insulin-related CRC and HCC.

Conflicts of Interest

We declare that no competing interests exist.

Acknowledgements

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