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World J Gastroenterol 2003;9(1):73-78
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

Synergetic anticancer effect of combined quercetin and recombinant adenoviral vector expressing human wild-type p53, GM-CSF and B7-1 genes on hepatocellular carcinoma cells *in vitro*

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Received: 2002-07-26 **Accepted:** 2002-08-16

Abstract

AIM: This study investigated the anti-cancer effect of combined quercetin and a recombinant adenovirus vector expressing the human p53, GM-CSF and B7-1 genes (designated BB-102) on human hepatocellular carcinoma (HCC) cell lines *in vitro*.

METHODS: The sensitivity of HCC cells to anticancer agents was evaluated by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The viability of cells infected with BB-102 was determined by trypan blue exclusion. The expression levels of human wild-type p53, GM-CSF and B7-1 genes were determined by Western blot, enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis, respectively. The apoptosis of BB-102-infected or quercetin-treated HCC cells was detected by terminal deoxynucleotidyl transferase (TdT) assay or DNA ladder electrophoresis.

RESULTS: Quercetin was found to suppress proliferation of human HCC cell lines BEL-7402, HuH-7 and HLE, with peak suppression at 50 μmol/L quercetin. BB-102 infection was also found to significantly suppress proliferation of HCC cell lines. The apoptosis of BB-102-infected HCC cells was greater in HLE and HuH-7 cells than in BEL-7402 cells. Quercetin did not affect the expression of the three exogenous genes in BB-102-infected HCC cells ($P>0.05$), but it was found to further decrease proliferation and promote apoptosis of BB-102-infected HCC cells.

CONCLUSION: BB-102 and quercetin synergetically suppress HCC cell proliferation and induce HCC cell apoptosis, suggesting a possible use as a combined anti-cancer agent.

Shi M, Wang FS, Wu ZZ. Synergetic anticancer effect of combined quercetin and recombinant adenoviral vector expressing human wild-type p53, GM-CSF and B7-1 genes on hepatocellular carcinoma cells *in vitro. World J Gastroenterol* 2003; 9(1): 73-78

http://www.wjgnet.com/1007-9327/9/73.htm

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most malignant diseases known. Surgical resections are incapable of removing all HCC cells and the disease is very resistant to anticancer agents, making chemotherapy an equally ineffective option[1]. Therefore, anti-tumor gene therapy strategies seem a good future option for the treatment of HCC[2]. Numerous studies have shown that tumor occurrence and progression are often related to mutations in tumor suppressor genes or loss of host anti-tumor immunity[3-9]. Therefore, a promising approach is transfer of tumor suppressor genes to cause tumor cell growth arrest or apoptosis, combined with cytokine genes to induce an effective immune response directly against both the genetically modified and the parental tumor cells[10].

In HCC, mutation or loss of tumor suppressor gene p53 is associated with malignancy and chemoresistance[11-13], making the wild-type p53 (WT-p53) gene a good candidate for replacement by gene therapy. Granulocyte-macrophage colony stimulating factor (GM-CSF) has been proven to be a potent, long-lasting inducer of antitumor immunity that promotes maturation of dendritic cells and enhances the function of antigen-presenting cells and natural killer (NK) cells[14-17]. In addition, the expression of members of the B7 gene family has been shown to be important in antitumor responses in both mice and humans[18-20]. However, most tumor cells, including HCC cells, lack B7-1 molecules on their surface, and as a result they escape recognition by the immune system[21]. Kim *et al*. found an increased antitumor effect with the combined expression of GM-CSF and B7-1 in athymic nude mice, suggesting that coexpression of GM-CSF and B7-1 may enhance antitumor activity[22]. Recombinant adenovirus vector BB-102, which expresses p53, GM-CSF and B7-1 genes[23], has been shown to inhibit the growth of various human carcinoma cells, such as hepatocellular carcinoma, lung cancer[24], and laryngeal cancer[25], and enhances carcinoma cell chemosensivity to anti-cancer agents.

Quercetin (3,3' ,4' ,5,7-pentahydroxyflavone) is one of most widely distributed bioflavonoids in the plant kingdom and is a common component of most edible fruits and vegetable. Humans consume approximately 1 g of dietary flavonoid daily. This compound has been shown to inhibit the growth of various human cancer cell lines[26-34], including leukemia, hepatocellular carcinoma, and estrogen-receptor positive breast carcinoma MCF-7, suggesting that quercetin may have anti-cancer and anti-metastasis potential[35-39]. The growth inhibitory effect of quercetin on tumor cells is found to be consequence of its ability to interfere with the enzymatic processes involved in the regulation of cellular proliferation: DNA, RNA and protein biosynthesis[40-43]. Quercetin also has shown to down-regulate or inhibit the phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP) kinase activities in human carcinoma cells, leading to a marked reduction of second messengers IP3 concentration and cell death[44,45]. Therefore, quercetin may be useful in the treatment of carcinomas with increased or down-regulated signal transduction capacity[45-47].

These results suggest that the combination of quercetin with BB-102 may synergetically suppress the growth of carcinoma cells. Accordingly, this study addressed the synergetic action of quercetin combined with BB-102 against HCC cells *in vitro*.

MATERIALS AND METHODS

Agents

Quercetin was purchased from Sigma (St Louis, MO). Western blot detection system ECL+plus Kit was purchased from Amersham Pharmacia Biotech (Arlinton Heights, IL). GM-CSF ELISA kit was bought from Genzyme (Cambridge, MA). The TdT FragEL™ DNA fragmentation detection kit was from Calbiochem (Cambridge, MA). Ad-GFP (Ad5 vector expressing green fluorescence protein) was kindly provided by the Gene Therapy Unit, Baxter Healthcare Corporation, USA. BB-102 was reconstructed by Dr ZH Qu. Quercetin was dissolved in medium containing 0.1 % dimethyl sulfoxide (DMSO), and the same concentration of DMSO was used in control experiments.

Quercetin inhibits the growth of HCC cells

The BEL-7402, HLE and HuH-7 cell lines were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were exposed to various concentrations of quercetin from 3.125 μmol/L to 100 μmol/L. After 72 h, the number of viable cells was determined by MTT assay.

Growth suppression in BB-102-infected HCC cells

HCC cell lines were seeded in six-well plates at a density of 5×10^5 cells/well. Twelve hours later, the cells were infected with BB-102 and Ad-GFP at a MOI of 50 pfu/cell. Culture medium was used for mock infection. Triplicate wells of each treatment group were counted every 2 days for a total of four times after infection. The viability of the cells was determined by trypan blue exclusion.

Assay of apoptosis

Apoptosis induced by BB-102 was analyzed by terminal deoxynucleotidyl transferase (TdT) assay. Briefly, HCC cells were seeded in 12-well plates at a density of 5×10 cells/well. After 24 h, they were infected with BB-102 or Ad-GFP at a MOI of 50 pfu/cell. Culture medium was used for mock infection. Seventy-two hours later, cell monolayers were fixed with 4 % formaldehyde diluted in phosphate-buffered saline (PBS) for 15 min at room temperature. Apoptosis of the cells was detected using a TdT FragEL™ DNA fragmentation detection kit according to the protocol.

Cellular apoptosis induced by quercetin or quercetin combined with BB-102 was analyzed by agarose gel-electrophoresis as described based on the pattern of DNA cleavage. Briefly, cells (1×10^6) were lysed with 0.5 ml lysis buffer, followed by the addition of RNase A to a final concentration of 200 μg/ml, and incubated for 1 h at 37 ℃. Cells were then treated with 300 μg/ml of proteinase K for 1 h at 37 ℃. After addition of 4 μl loading buffer, 20 μl samples in each lane were subjected to electrophoresis on a 1.5 % agarose at 50 V for 3 h. DNA was stained with ethidium bromide and laddering was visualized under UV light.

P53 gene expression by western blot

Three cell lines were seeded in 6-well plates at a density of 1×10^5 cells/well. Group II was treated with 100 μmol/L quercetin, group III was infected with BB-102 at a MOI of 50 pfu/cell, group IV was infected with BB-102 and then treated with 100 μmol/L quercetin, and group I was mock-infected with culture medium infected with empty adenovirus vector as control. After 48 h incubation, cells were washed with phosphate buffered saline, disrupted by addition of lysing buffer (100 mmol/L Tris· Cl pH 6.8,200 mmol/L DTT,4 %SDS, 0.2 % bromophenol blue, 20 % glycerol), and electrophoresed. Western blot detection system ECL+plus Kit was used to determine p53 expression according to the instruction manual.

ELISA of GM-CSF gene expression

Expression of human GM-GSF was detected by enzyme-linked immunosorbent assay. Briefly, the cells were seeded in 6-well plates at a density of 1×10^6 cells/well. After 24 h, the cells were infected with BB-102 at a MOI of 50 pfu/cell for 1 h, then BB-102 suspensions were replaced with either culture medium alone, or culture medium containing 100 μmol/L quercetin. After 48 h, the suspension was collected. The GM-CSF present in each suspension was quantified using the ELISA kit according to the protocol.

FCM of B7-1 gene expression

HCC cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After 24 h, the cells were either treated with 100 μmol/L quercetin, infected with BB-102 at a MOI of 50 pfu/cell, or infected with BB-102 at a MOI of 50 pfu/cell then treated with 100 μmol/L quercetin. Culture medium was used for mock infection. After 48 h incubation, the cells were collected. Cells were washed twice in PBS and resuspended in PBS containing 1 % bovine serum albumin (BSA) prior to incubation with anti-human B7-1 monoclonal antibody (MoAb) for 30 min at 4 ℃ in the dark, followed by two washes in PBS/BSA. The cells were stained with FITC-conjugated goat anti-mouse IgG for 30 min at 4 ℃. Nonspecific binding was controlled by incubation with isotypic controls. Fluorescence was measured with FACSCalibur flow cytometer (Becton Dickinson).

Proliferation of cells treated with both quercetin and BB-102

HCC cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were infected with Ad-GFP or BB-102 at a MOI of 50 pfu/cell. Culture medium was used for mock infection. After 48 h, cells were treated with various concentrations of quercetin from 3.125 to 50 μmol/L. Culture medium was used for mock treatment. After 72 h, the viable cell numbers were tested by MTT assay.

RESULTS

Antitumor effect of quercetin on HCC cells

Quercetin was shown to inhibit HCC proliferation and induce HCC apoptosis *in vitro* in a dose-dependent manner until it reached peak inhibition at 50 μmol· L^{-1} (Figure 1).

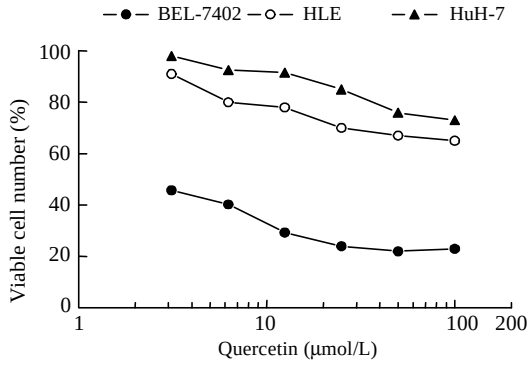

Figure 1 The suppressive effect of quercetin on the proliferation of BEL-7402, HLE and HuH-7 cell lines *in vitro.*

BB-102 inhibit the growth of HCC cells

Introduction of p53, GM-CSF and B7-1 through BB-102 infection led to significant suppression of growth proliferation in BEL-7402, HLE and HuH-7 cells compared with those infected with Ad-GFP or mock infected (Figure 2). Among them, there was a lower suppression in BEL-7402 cell line compared with HLE and HuH-7 cell lines ($P<0.05$), which might be related

to the fact that BEL-7402 expresses an endogenous wild-type p53, while HLE and HuH-7 both express endogenous mutant p53, resulting in BEL-7402 being less sensitive to the effects of the exogenous p53 protein expressed by BB-102.

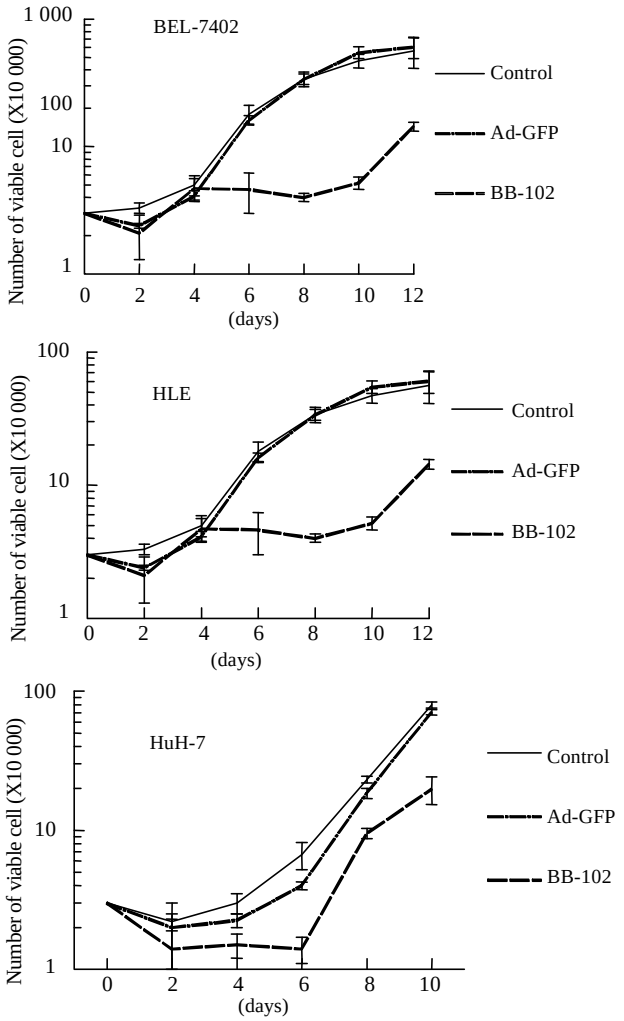

Figure 2 Proliferation inhibition of HCC cell lines transduced with BB-102.

BB-102 induces HCC cells apoptosis

As measured by TdT assay, apoptotic rates in BEL-7402, HLE and HuH-7 cells lines were were 12.75 %, 57 % and 49.5 %, respectively. This suggests that HLE and HuH-7 cells were more sensitive to BB-102 than BEL-7402 cells.

Quercetin induces apoptosis of HCC cells

DNA laddering (Figure 3) suggests that quercetin induces HCC cell apoptosis, and that BB-102 further promoted the quercetin-treated cell apoptosis.

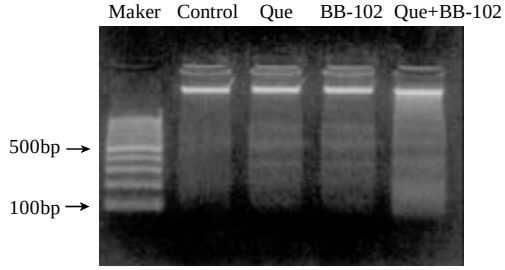

Figure 3 The promotion of Que on HCC cell apoptosis induced by BB-102.

Quercetin does not affect exogenous gene expression in BB-102-infected cells

Expression of exogenous P53 in BB-102-infected cells By using Western blot analysis, high levels of p53 protein were detected in BB-102-infected HCC cell lines. These levels were not affected by treatment with quercetin (Figure 4).

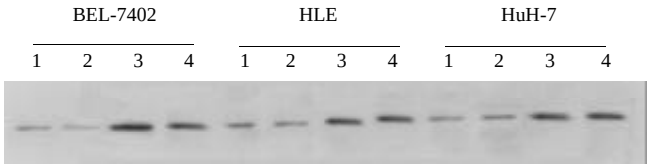

Figure 4 Effect of Que on the p53 expression in HCC cell lines transfered with BB-102. (lane1: control; lane2: with Que; lane3: with BB-102; lane4: with Que and BB-102).

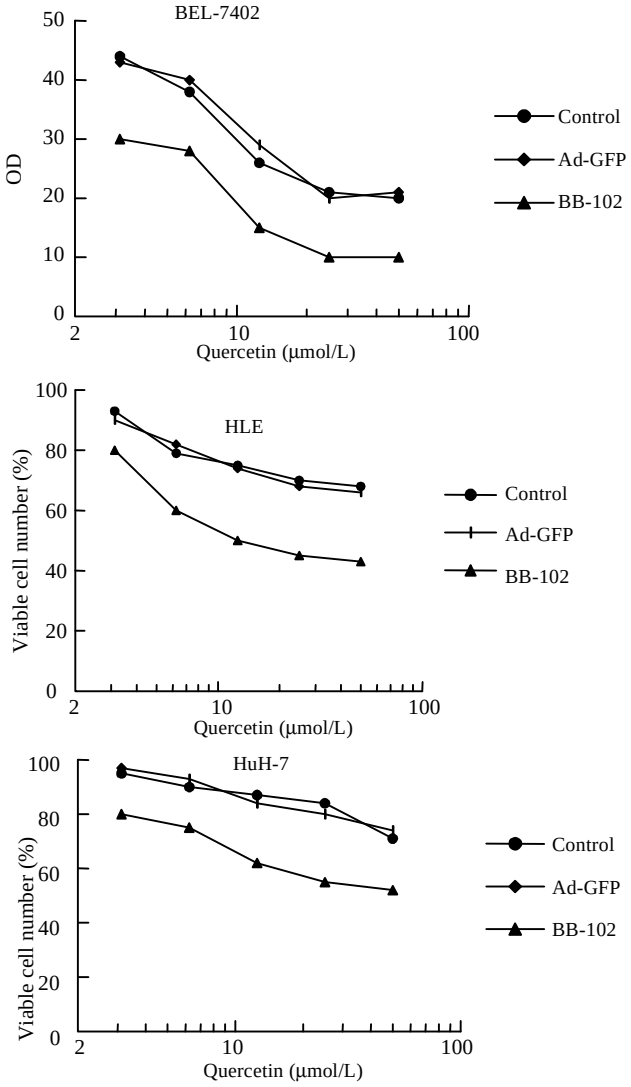

Figure 5 Synergetic effect of BB-102 on quercetin suppression of growth of HCC cell lines.

Expression of exogenous GM-CSF protein in BB-102-infected cells ELISA assay showed that a high-level expression of GM-CSF was detected in the culture suspension of BB-102-infected cells 48 h after infection. There was no statistical difference in GM-CSF level between the cells treated with quercetin and untreated cells (Table 1).

Expression of exogenous B7-1 protein in BB-102-infected cells B7-1 gene expression was observed flow cytometric analysis 48 h after BB-102 infection (Table 2). There was no

significant difference in B7-1 expression between cells treated with quercetin and untreated cells.

Table 1 Effect of Que on the GM-CSF expression in HCC cell lines transferred with BB-102 ($pg \cdot L^{-1}$), ($\bar{x}\pm s$), (n=6)

Cell line	Infected with BB-102	Infected with BB-102+Que
BEL-7402	118.9±29.9	147.7±13.2
HLE	209.6±55.7	248.3±15.9
HuH-7	250.7±21.9	283.7±21.6

Table 2 Effect of Que on the B7-1 expression in HCC cell lines transfered with BB-102 (%) (n=4)

Cell line	Control	Que	BB-102	Que+BB-102
BEI-7402	10.0±2.5	11.2±1.7	80.0±10.3[b]	85.0±12.0[b]
HLE	15.3±2.1	14.0±2.0	31.0±7.8[a]	35.2±5.6[b]
HuH-7	20.0±2.6	22.0±3.0	59.8±8.5[b]	64.0±8.8[b]

[a]P<0.01, [b]P<0.001, *vs* their controls

Quercetin enhances the inhibition of the proliferation in BB-102-infected HCC cell lines

The proliferation suppression pattern of HCC cells treated with quercetin was not significantly different between Ad-GFP-infected cells and Ad-mock infected cells. In contrast, BB-102-infected cells showed a significant increase of suppression when treated with quercetin. This implies that BB-102 transduction and quercetin treatment synergistically suppress HCC proliferation (Figure 5). The control data indicates that it was the exogenous gene expression that induced this effect, not the adenovirus.

DISCUSSION

Gene therapy, the introduction of functional genes into cells to treat or prevent a disease, is a promising approach for the treatment of human cancer. In an effort to explore potential gene therapy approaches to the treatment of stubborn HCC, we investigated the synergetic effects of dietary component (quercetin) plus gene transfer (BB-102 infection). BB-102 is a recombinant adenoviral vector expressing the genes for human wild-type p53, GM-CSF and B7-1[23]. Our study showed that BB-102 infection arrested the growth of HCC cells lines BEL-7402, HLE, HuH-7, and induced their apoptosis by the expression of exogenous wild-type p53 (a transcriptional activator that induces cell cycle arrest and apoptosis[48-50]). BB-102 also enhanced immunogenicity of HCC cells and improved host antitumor immune reaction.

We then examined the possible synergy between these exogenous genes and quercetin, which is thought to have a long-term preventive effect on chemical carcinogenesis, especially in people who eat a diet rich in fruits and vegetables[30,31,36,51,52]. Previously, we had found that antineoplastic drug concentrations exerting cytotoxic activity were markedly lower when cells were pretreated with quercetin[28,35]. In addition, quercetin has been shown to inhibit the growth of human breast cancer cell line MDA-MB468 in a dose-dependent fashion by specifically inhibiting the expression of mutant p53 in cellular transformation[53]. Because HCC patients are generally resistant to chemotherapy (possibly due to the loss of a functional p53 gene[54], which is known to occur in 50 % of cancers[55-58]), we questioned whether quercetin could reverse the multi-drug resistance of HCC cells, perhaps through down-regulation of mutated p53 or glycoprotein[53,59,60]. In addition to its possible role in adjusting p53 levels, quercetin is also known to inhibit the synthesis of HSP70 (heat shock protein 70) and change its intracellular distribution. HSP70 is involved in apoptosis, and the link between the two suggests that quercetin may be involved in the induction of apoptosis[35,61].

We found enhancement of apoptosis and inhibition of the proliferation of BB-102-infected HCC cell lines treated with quercetin, suggesting that there is a synergetic anticancer effect between quercetin and BB-102. There was no change in p53, GM-CSF and B7-1 gene expressions in BB-102-infected HCC cell lines following quercetin treatment, indicating that quercetin did not affect exogenous gene expression in BB-102-infected HCC cells. We believe it is possible that this synergetic effect might be due to BB-102 transduction promoting the chemosensitivity of HCC cells to quercetin, but this and other mechanisms will need further study.

BEL-7402 cells were more sensitive to quercetin than HLE and HuH-7 cells. At a concentration of 50 umol/L quercetin (peak proliferation inhibition), proliferation inhibition was 78 %, 33 % and 24 % for BEL-7402, HLE and HuH-7, respectively. Our previous studies have shown that the doubling time of BEL-7402 is the shortest among these cells. This suggests that the cells that proliferate quickly are more sensitive to quercetin in this study. In addition, quercetin induced apoptosis in HCC cells at G1 and S in a dose- and time-dependent manner, and the effect was enhanced by BB-102 infection. This enhancement was less in BEL-7402, possibly due to its wild-type endogenous p53 status[62]. Overall, HCC cells treated with both quercetin and BB-102 showed inhibition of proliferation and increased apoptosis, suggesting that quercetin may be useful as an adjuvant in chemotherapy treatment of HCC patients. The combination of quercetin and BB-102 transduction is a promising new strategy for the treatment of typically chemo-resistant hepatomas.

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Edited by Wu XN