Full-length article

Antisense oligonucleotides targeting midkine induced apoptosis and increased chemosensitivity in hepatocellular carcinoma cells

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Abstract

Aim: Overexpression of midkine (MK) has been observed in many malignancies. This aim of this study is to screen for suitable antisense oligonucleotides (ASODN) targeting MK in hepatocellular carcinoma (HCC) cells and evaluate its antitumor activity. **Methods:** Ten ASODN targeting MK were designed and synthesized. After transfection with ASODN, cell proliferation was analyzed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. In addition, MK mRNA, protein levels, as well as apoptosis and caspase-3 activity were also examined in HepG2 cells. Cell proliferation was then analyzed after treatment with both ASODN and chemotherapeutic drugs. **Results:** In this experiment, the ASODN5 among the 10 ASODN showed higher inhibitory activity against proliferation of hepatocellular carcinoma cells in a dose-dependent manner. In HepG2 cells, ASODN5 could significantly reduce the MK mRNA level and protein content. After transfection with ASODN5 for 48 h, accompanied with a decline of survivin and Bcl-2 protein content, a remarkable increase of apoptosis and caspase-3 activity was observed in HepG2 cells. Furthermore, ASODN5 transfer can significantly increase chemosensitivity in HepG2 cells. **Conclusion:** Antisense oligonucleotides targeting MK shows therapeutic effects on HCC; ASODN5 has the possibility to be developed as an effective antitumor agent.

Introduction

Midkine (MK) was first identified in embryonal carcinoma cells in the early stages during retinoic acid-induced differentiation[1]. MK and pleiotrophin (also known as the heparin-binding growth-associated molecule) comprise a family of heparin-binding growth/differentiation factors, different from other heparin-binding growth factors such as fibroblast growth factor and hepatocyte growth factor[2–4]. It has been reported that MK is overexpressed in various malignant tumors, such as lung, breast, esophageal, gastric, colorectal, liver, pancreatic, ovarian, urinary bladder, prostatic, cerebral and renal malignancies[5–13], whereas in normal adult tissues, MK is low or undetectable[14]. High expression of MK is correlated with poor prognosis for patients with urinary bladder carcinomas, glioblastomas and neuroblastomas[9,11,12].

Elevated blood MK has also been reported for esophageal cancer, neuroblastoma and gastric cancer[15–17]. MK promotes the survival[18,19], growth[20,21] and migration[22,24] of many cells, which in combination may contribute to oncogenesis and tumor progression. MK can activate mitogen-activated protein kinase (MAPK) pathways through inducing the phosphorylation of p44/42 MAPK (Thr202/Tyr204), subsequently promoting cell growth[25]. MK also activates extracellular signal-regulated kinases 1 and 2, which are well known as signal transducers acting downstream several receptors[18]. The activated MAPK pathway was reported to downregulate caspase-3 activity in neurons[25]. In addition, it was also reported that MK can induce phosphorylation of protein kinase B(AKT) (Ser473 and Thr308). The phosphorylated AKT then promotes a series of anti-apoptosis pathways in cells.

Hepatocellular carcinoma (HCC) is one of the most common malignancies. Several mechanisms of carcinogenesis have been proposed for HCC[26,29]. Downregulation of apoptosis and accelerated proliferative activity in hepatic cells have been regarded as malignant factors leading to HCC[30]. Recently, HCC tumor cells were found to overexpress MK[31]. These findings suggest that MK could be a potential target for hepatocellular carcinoma therapy.

By inducing degradation of the mRNA encoding the protein, oligonucleotides have the potential for cancer therapy[32]. The present study aims to develop potential antisense oligonucleotides (ASODN) to repress MK expression in hepatocellular carcinoma cells and examine their antiproliferation activity.

Materials and methods

Antisense oligodeoxynucleotides All the antisense compounds used in these experiments were designed by a computational neural network model[33]. BLAST confirmed that they are specific for MK mRNA. 20 mer ASODN were synthesized using an applied Biosystems 391 DNA synthesizer and purified by OPC (Perkin-Elmer, Foster City, CA, USA). Table 1 shows the sequence of ASODN.

Cell lines and transfection Human liver hepatocellular carcinoma cell lines (HepG2, SMMC-7721 and BEL-7402 were from the Chinese Academy of Medical Science, Beijing, China) were grown in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI-1640 medium (Invitrogen Corporation, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, and 5% CO_2. 3×10^3 cells were seeded in each well of a 96-well microtiter plate and allowed to attach overnight. Oligonucleotides with concentrations of 0.1, 0.2, 0.4, 0.8 μmol/L were transfected into the cells with Lipofectin (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Transfection medium was replaced by normal culture medium after 6 h.

Cell viability assay The effects of ASODN on cellular viability were measured by MTS (3-[4, 5-dimethythiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium, inner salt) assay. After 48 h of incubation following transfection, 20 μL MTS (Sigma, St Louis, MO, USA) was added to each well and incubated at 37 °C for 2 h. The absorbance value was determined at 490 nm by a MR600 Microplate reader (Wallac 1420 Multilable counter, Wallac, Turku, Finland).

To investigate whether the transfection of ASODN enhances chemosensitivity of cancer cells, HepG2 cells were treated by combination of chemotherapeutics (5-fluorouracil (5-FU), adriamycin (ADM) and *cis*-diaminodichloroplatin (DDP)) and ASODN5 at various concentrations. Cell proliferation was analyzed after 24 h as described earlier. Each assay was performed in quadruplicate. The proliferation inhibition rate was calculated as: $(A_{control}-A_{sample})/(A_{control}-A_{blank})\times100$.

RT-PCR The total RNA from cell lines was extracted using Trizol (Invitrogen, Carlsbad, USA) by a single-step phenol extraction. Subsequent RT-PCR reaction was performed using an RT-PCR kit (Promega, Madison, WI, USA). PCR products were run on 2.0% agarose gel and visualized by ethidium bromide staining. The product intensities were measured by scanning the gel with Gel Doc 1000 (Bio-Rad, Hercules, CA, USA), with GAPDH serving as an internal control.

Western blot analysis The cells in the microplates were collected into 1.5 mL eppendorf tubes in ice and centrifuged at 2000 r/min for 5 min at 4 °C. They were then resuspended in 100 μL cell lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-Cl, pH 8.0, and 4% SDS), and incubated on ice for 1 h to lyse the cells. The lysates were centrifuged at 14 000 r/min for 15 min at 4 °C; the supernatants were collected and stored at –20 °C for electrophoresis. An extract of each sample was separated on 12% sodium dodecyl sulfate polyacrylamide electrophoretic gels. The separated proteins were blotted onto Hybond-polyvinylidene difluoride membranes (Schleicher & Schuell Biosciences, Inc, Keene N H, USA) and incubated in 5% skimmed milk blocking solution at room temperature overnight. The reactive band was visualized with an ECL-plus Detection Kit (Amersham Biosciences, Piscataway, NJ) and scanned by Gel Doc 1000 (Bio-Rad CA, USA). β-actin was used as a control.
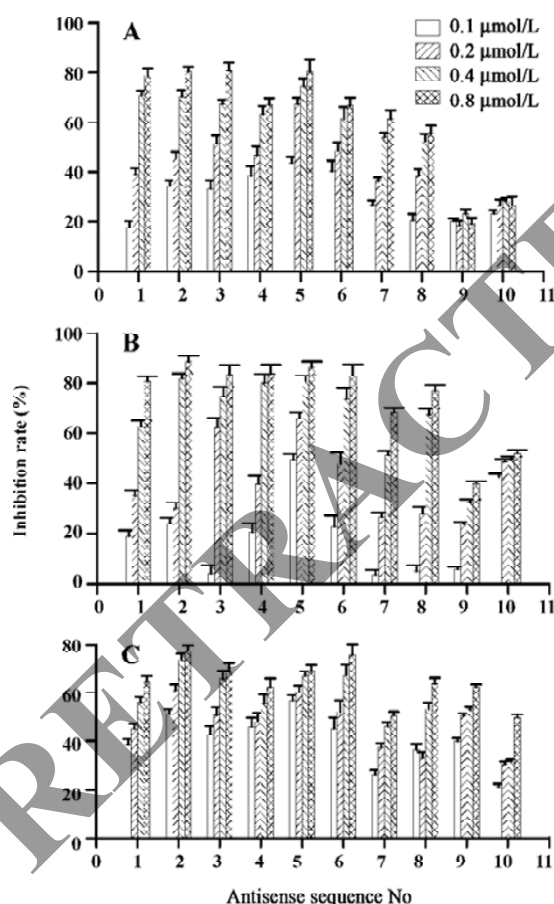
Measurement of apoptosis and caspase-3 activity analysis Apoptosis was assessed for the ASODN5-transfected cells after culture in normal medium for 48 h using Annexin V-FITC/PI (Baosai, Beijing, China) according to the manufacturer's protocol. Flow cytometry was performed on a FACScalibur machine and the data was analyzed using CellQuest software (SOBR model, Becton Dichinson Instrument, San jose, CA, USA). Caspase-3 activity was measured using the colorimetric protease assay kit according to the manufacturer's protocol (Calbiochem, La Jolla, CA, USA). HepG2 cells were cultured in 100 mm dishes in DMEM medium plus 10% FBS for 24 h. ASODN5 0.4 μmol/L was added. Cytosol extracts of the cells were diluted to a concentration of 100 μg protein per 50 μL cell lysis buffer. Protease activity was measured using the tetrapeptide *p*-nitroanilide substrate. The absorbance was measured at
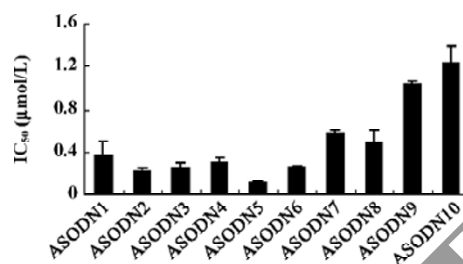
405 nm.

Statistics Data were expressed as mean±SD. Statistical analysis was carried out using Student's-*t*-test (two tailed); $P<0.05$ indicated statistical significance.

Results

Effects of ASODN on hepatocellular carcinoma cell proliferation The 10 ASODN designed to target against MK were individually screened for their abilities to inhibit hepatocellular carcinoma cell proliferation. All 10 ASODN showed various inhibitory activities on the 3 lines of cancer cells, BEL7402, HepG2, and SMMC-7721. The inhibition rate ranged from 20% to 85%, positively related to the ASODN concentrations (Figure 1). Among the 10 ASODN, ASODN1–6 showed higher inhibitory activity. Meanwhile, we also observed that ASODN6, ASODN5, ASODN3, and ASODN2 had lower IC_{50}, respectively (Figure 2). We therefore choose

Figure 2. The IC_{50} of ten ASONDs in hepatocellular carcinoma cells (n=3).

the 4 ASODN for further experiments.

Effects of ASODN on MK expression Since ASODN6, ASODN5, ASODN3 and ASODN2 had lower IC_{50} respectively, we wanted to determine whether they would decrease MK expression in HepG2 cells. From Figure 3, we obtained that only ASODN5, ASODN3 and ASODN2 downregulated the MK mRNA level significantly in our experiment. Given the lowest IC_{50} (0.11 μmol/L), we then selected ASODN5 as a candidate for antitumor drug study. To further characterize the function of ASODN5 on MK expression (mRNA and protein), HepG2 cells highly expressing MK were transfected with ASODN5 (0.4 μmol/L), whereas
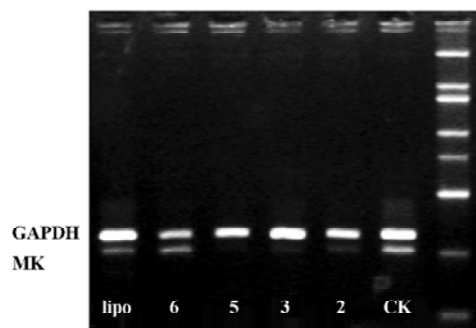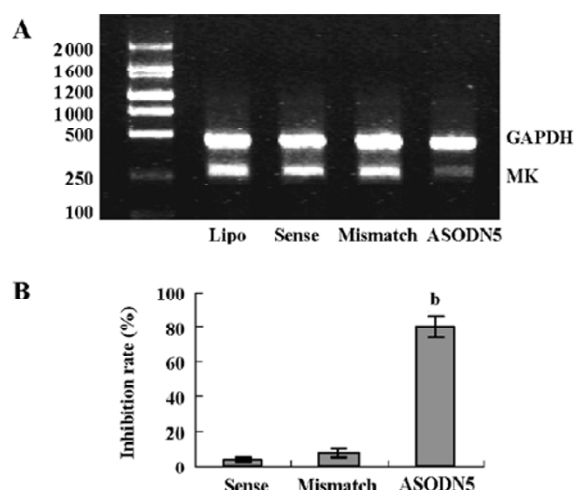

Figure 1. Effects of ASODNs on the proliferation in 7402 (A), HepG2 (B), and 7721(C) cells. Cell proliferation was measured after transfection of ASODN for 48 h. Data were expressed as means±SD from four independent experiments.
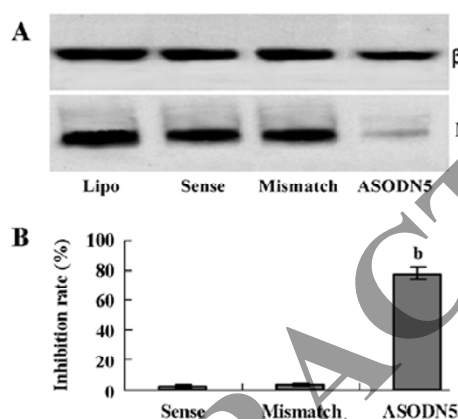

Figure 3. Effect of ASODN6, ASDON5, ASDON3, and ASDON2 on MK mRNA level. GAPDH was used as a control.

the cells transfected with mismatched compound (0.4 μmol/L), sense compound (0.4 μmol/L) and Lipofectin (0.4 μmol/L) were used as controls. Forty-eight hours after transfection, the cells were examined by RT-PCR and Western blotting. As shown in Figure 4, ASODN5 lowered the MK mRNA level in HepG2 cells, with mismatched, sense compound (0.4 μmol/L) and Lipofectin (0.4 μmol/L) showing no effect. In addition, the MK protein level was decreased by 78.2% ($P<0.05$) in HepG2 cells by the ASODN5 (Figure 5). Data presented here

Figure 6. Effect of ASODN5, mismatch and sense oligonucleotides at 0.1–0.8 µmol/L on cell proliferation of HepG2 cells. Cell proliferation was measured after transfection with ASODN for 48 h. Data were expressed as means±SD from four independent experiments
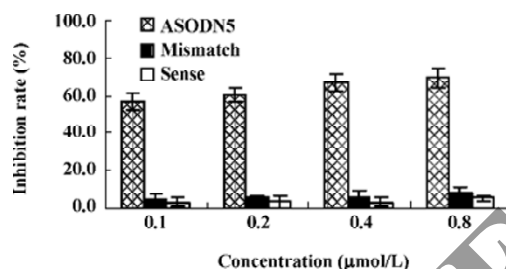
Figure 4. Effects of oligonucleotides on mRNA level of MK in HepG2 cells. A: Electrophoresis of RT-PCR products of MK gene and GAPDH gene in HepG2 cells transfected with ASODN5, Mismatch and Sense; B: Quantitation of the data presented in (A). Data were expressed as means±SD from four independent experiments. [b]$P<0.05$ *vs* the cells (CK) transfected with lipofectin alone.

experiment, the caspase-3 activity increased in the ASODN5-treated cells (Figure 7A). Interestingly, the survivin and Bcl-2 protein content decreased with MK antisense compound transfection in HepG2 cells (Figure 7B). Since the ASODN5 can downregulate caspase-3 activity, then flow cytometry was then used to quantify the changes in cell apoptosis after transfection with ASODN5 for 48 h. From Figure 8, it was clear that there was about 38.0%±2.5% (*n*=3) cell entry apoptosis after transfection with ASODN5.

Figure 5. Analysis of MK protein expression after transfection with ASODN5, mismatch and sense. HepG2 cells were cultured in DMEM medium. A: Cell extracts were separated by SDS gel electrophoresis, transferred to a membrane, and blotted with anti-MK goat polyclonal antibody. Antiactine rabbit polyclonal antibodies was used as a loading control. B: Quantitation of the data presented in (A). Data were expressed as means±SD from four independent experiments. [b]$P<0.05$ *vs* the cells (CK) transfected with lipofectin alone.

Figure 7. Analysis of caspase-3 activity(A), Bcl-2 and survivin protein content (B) in HepG2 cells treated with 0.4 µmol/L sense, mismatch and ASODN 5 targeting MK compounds for 48 h. Enzyme activity was measured at 405 nm. [b]$P<0.05$ *vs* the cells (CK) transfected with lipofectin alone.
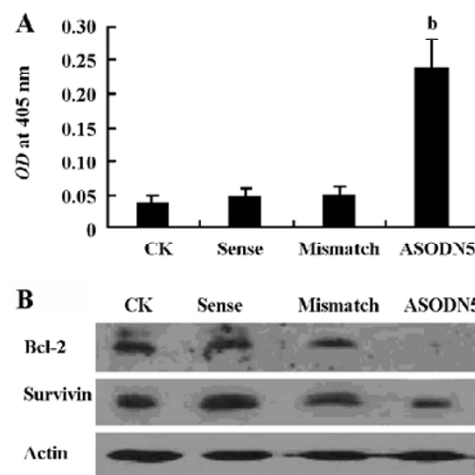
suggests that ASODN5 can specifically downregulate MK expression. Additionally, mismatched and sense compounds showed little effect on cell proliferation in HepG2 cells (Figure 6).

Analysis of cell apoptosis and caspase-3 activity It has been reported that MK protects cells against apoptosis through downregulating caspase-3 activity[35]. In this

Inhibition of cell proliferation after combination with chemotherapeutic drugs Figure 9 shows the results of combination therapy with chemotherapeutic drugs. It is clear that chemotherapeutic drugs 5-Fu, ADM and DDP can significantly reduce HepG2 cell growth, and the inhibitory
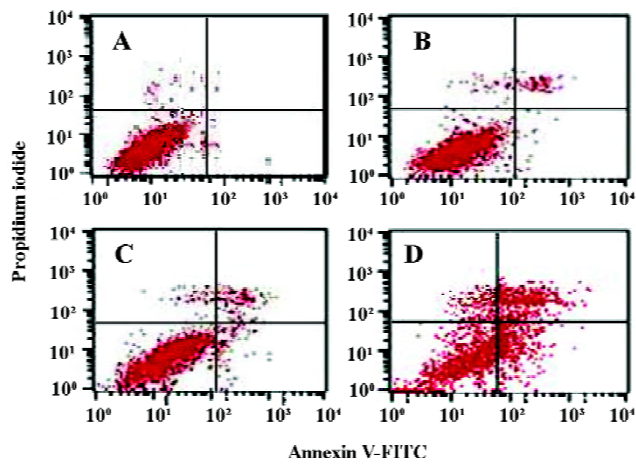
Figure 8. Analysis of HepG2 cells apoptosis after treated with 0.4 μmol/L sense, mismatch and ASODN5 for 48 h by staining with Annexin-V-FITC and PI and analyzing by flow cytometry. A: control; B: sense; C: mismatch; D: ASODN5.

effect exhibited a dose-dependent manner. It is interesting to observe that ASODN5 transfer can increase the chemosensitivity of 5-Fu, ADM and DDP. It is noted that the synergis-

tic cytotoxic effect is more effective at low concentrations.

Discussion

Accumulating data suggest that MK, a heparin-binding growth factor, is overexpressed in a wide range of human carcinomas and is believed to contribute to tumorigenesis and tumor progression. Antisense approaches are efficient tools for the inhibition of gene expression in a sequence-specific way. However, it is known that the screen of high active ASODN is difficult because of the complexity of the RNA structure[35]. In the present study, we generated sequence-specific antisense compounds to downregulate MK expression (mRNA and protein) in hepatocellular carcinoma cells leading to the inhibition of their growth.

In this study, we first designed antisense sequences using a neural network tool, and several candidate sequences were obtained. In order to improve the effectiveness of ASODN, the MK mRNA secondary structure was also predicted according to Zuker[36]. Using this theoretical approach, 10 putative target sites were identified (Table 1). Our data show that all of the 10 ASODN have inhibitory
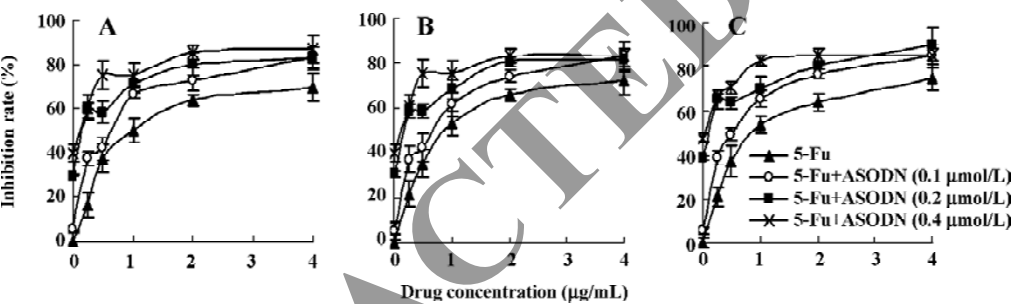
Figure 9. Cell proliferation of HepG2 cells after combination treatment with ASODN5 and chemotherapeutic drugs (A: 5-Fu; B: ADM; C: DDP). Experiments were carried out after combination treatment for 24 h.

Table 1. The sequence of ASODNs

No	Name	Base position	Size (nt)	Sequence (5'–3')
1	ASODN 1	629–648	20	ATCCCTTGTCCCTCCCCACT
2	ASODN 2	178–197	20	CGCGGAAACCCACGCCGCAA
3	ASODN 3	1–20	20	TGCCCGCGCTGCTTGGCCCG
4	ASODN 4	428–447	20	CCCTTCCCTTTCTTGGCTTT
5	ASODN 5	108–127	20	CCCCGGGCCGCCCTTCTTCA
6	ASODN 6	296–315	20	CCCCATCACACGCACCCCAG
7	ASODN 7	435–454	20	GTCCTTTCCCTTCCCTTTCT
8	ASODN 8	636–655	20	TCCCAGAATCCCTTGTCCCT
9	ASODN 9	61–80	20	AGGAGGGTGAGGAGGAGGAA
10	ASODN 10	511–530	20	CGGGCCTGGGAGAGGGAGGG
11	Sense	—	20	TGAAGAAGGGCGGCCCGGGG
12	Mismatch	—	20	CCGGCCGCGCGGCAAGATCA

activity, and ASODN5 has the lowest IC_{50} at 0.11 μmol/L. The result suggests that the computational method is a good approach for finding putative target sites.

As measured by RT-PCR and Western blotting, the antisense compounds efficiently downregulated the MK expression level in HepG2 cells in a dose-dependent manner. However, the transfection of mismatched and sense compounds showed little effect on MK expression in HepG2 cells (Figures 4,5). At the same time, ASODN5 transfection inhibited the proliferation of HepG2 cells significantly. Taken together, our data suggests that the proliferation inhibition by ASODN was due to the downregulation of MK expression.

Several mechanisms were proposed to increase cell proliferation by MK. Among these, the downregulation of caspase-3 activity is well recognized. Here the antisense compounds induced a strong growth-inhibitory effect and apoptosis on HepG2 cells. We also found that caspase-3 protease activity increased in cells transfected with antisense targeting MK (Figure 7A). Bcl-2 and the survivin protein were thought to be very important anti-apoptotic proteins in cells. Survivin can interact with the caspase-3 enzyme and lead to a reduction of caspase-3 enzyme activity[37]. Additionally, Bcl-2 can form a complex with Bax, and then suppress the activation of caspase-3[38]. Since MK antisense transfection can downregulate caspase-3 activity, we wanted to determine whether the declined caspase-3 activity is associated with survivin and Bcl-2. To our surprise, MK antisense transfection led to a marked decline of survivin and Bcl-2 protein expression (Figure 7B). We therefore speculated that the downregulation of survivin and Bcl-2 protein expression by MK antisense transfection in one of the important ways to activate caspase-3 activity, subsequently inducing cell apoptosis. Our data presented here also provide new anti-apoptotic mechanisms of MK. In short, these data suggest that the loss of MK expression is sufficient to trigger caspase-dependent apoptosis in HepG2 cells.

Drug tolerance is often developed during combination chemotherapy of HCC. Thus, the approach of enhancing chemosensitivity by using apoptosis-inducing agents appears to be a proper strategy for patients with advanced cancers. It is known that MK is overexpressed in liver hepatocellular cells and can restrain cell apoptosis through the downregulation of caspase-3 activity. Therefore, MK antisense transfer is anticipated to increase chemosensitivity. In our work, 3 chemotherapeutics (5-Fu, ADM and DDP) combined with ASODN5 targeting MK, increased chemosensitivity significantly. Downregulation of MK could be a promising strategy to reduce drug resistance of hepatocellular carcinoma.

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