Antitumor Effect of Zinc Acetate in Hepatocellular Carcinoma Cell Lines via the Induction of Apoptosis

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(Received February 17, 2022)

Summary We aimed to verify antitumor effects of zinc acetate on hepatocellular carcinoma (HCC) in vitro. Five HCC cell lines (HepG2, Hep3B, Huh7, HLE and Alex) were used to evaluate the antitumor effects of zinc acetate. Cell viability was determined by the Cell Counting Kit-8 assay. The cell-cycle alteration was evaluated by a flow cytometric analysis and the detection of cell cycle-related proteins. Apoptosis was determined based on the caspase-cleaved cytokeratin 18 (cCK18) levels. The microRNAs (miRNAs) related to an antitumor effect of zinc acetate were identified using microarrays. Zinc acetate significantly inhibited the proliferation of HCC cells in a dose-dependent manner. The treatment with zinc acetate resulted in significantly increased cCK18 levels in the supernatant and enhanced the expression of heme oxygenase-1 (HO-1) in HCC cells. The flow cytometric analysis revealed an increase of HCC cells in the S and G2/M phases by the administration of zinc acetate, and the expressions of Cdk2 and cyclin E were increased. The miRNA expression profile of the HCC cells treated with zinc acetate was extremely different from that of the untreated HCC cells. These results suggest that the zinc acetate supplementation induces the apoptosis of HCC cells, but does not affect the cell cycle progression. Upregulation of HO-1 and the alteration of miRNAs’ profile may be involved in antitumor effects of zinc acetate in HCC cells.

Key Words hepatocellular carcinoma, zinc acetate, antitumor effect, apoptosis, microRNA

It has been fully established that hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (1). Patients with HCC are often associated with underlying liver disease such as chronic hepatitis and liver cirrhosis. The treatment algorithm for HCC thus considers not only the size and number of tumors, vascular invasion, and distant metastasis, but also the liver functional reserve (2). A curative resection or locoregional treatments, including radiofrequency ablation or ethanol injection, can be selected for HCC patients whose hepatic reserve is favorable and whose tumor is localized in the liver, i.e., Barcelona Clinic Liver Cancer (BCLC) stage 0 or A. In contrast, systemic treatment by transarterial therapies is recommended for HCC patients who have intermediate-stage (BCLC stage B) with progressive disease or advanced-stage (BCLC stage C) (3).

No cytotoxic agents have been confirmed to have the beneficial effects on overall survival in advanced HCC patients, including doxorubicin, epirubicin, 5-fluorouracil and cisplatin. A standard cytotoxic regimen has not been established as a systemic treatment of HCC (4). The approval of the oral multikinase inhibitor, sorafenib for the treatment of HCC resulted in an improvement of overall survival (5), and thereafter, novel molecular-targeted agents, including regorafenib and lenvatinib, became available as systemic treatments for HCC, although the prognosis of the HCC patients remains unfavorable (6). Recently, several immune checkpoint inhibitors that restore T-cell-mediated anti-tumor activities have been tested in unresectable HCC patients (7), and combination therapy of a molecular target agent with an immune checkpoint inhibitor such as bevacizumab plus atezolizumab has been established as a first-line treatment for advanced HCC patients (8). Zinc is an essential trace element, and it is a key constituent or cofactor of over 300 mammalian proteins (9). It plays crucial roles in not only physical growth and development but also immune system function. Zinc also contributes to the function of transcription factors, anti-oxidant defense and DNA repair. Zinc deficiency can thus result in various of biological disorders, including the induction of DNA breaks and oxidative modification to DNA that initiates carcinogenesis (10). Indeed, the zinc content in the HCC tissue was signifi-
cantly lower compared to that in the liver tissue surrounding the tumor (11).

Zinc administration has resulted in the alleviation of hepatic activities (12) and the improvement of hepatic encephalopathy (13). Moreover, the administration of zinc compounds, including zinc sulfate, zinc chloride, zinc gluconate, and polaprezinc (a complex of zinc and L-carnosine), shows remarkable anti-tumor effects, by inducing apoptosis and/or cell cycle arrest, or by inhibiting tumor cell invasion and angiogenesis (14–22). However, no previous data are available on the antitumor effect of zinc acetate despite its efficacy in patients with Wilson’s disease (23) and those with liver cirrhosis (24).

The primary purpose of this study was to explore the antitumor effects of zinc acetate in vitro, using several HCC cell lines, and to reveal the putative mechanisms by which Zn acetate exerts antitumor effects. We also sought to identify any microRNAs (miRNAs) associated with the antitumor effect of zinc acetate.

MATERIALS AND METHODS

Chemicals. Zinc acetate dihydrate was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other chemicals were purchased from Sigma Chemicals (Tokyo, Japan). The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Cell lines and cultures. The live human HCC cell lines, HepG2, Hep3B, Huh7, HLE and Alex were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan) and were passaged in our laboratory. The HepG2 and Hep3B cells were grown in minimal essential media (MEM), and the Huh7, HLE and Alex cells in Dulbecco’s modified Eagle’s medium (DMEM), with both media supplemented with 10% fetal bovine serum (FBS) and 100 mg/mL of penicillin-streptomycin in a humidified atmosphere with 5% of CO2 at 37°C.

Cell proliferation essays. The cell proliferation assays were conducted using the CCK-8 and cultured in 100 μL of MEM or DMEM supplemented with 10% FBS. Cells from each cell line (0.2×104 cells) were seeded into a 96-well plate. Twenty-four hours later, the seeded cells were treated with 0, 40, 50, and 60 μg/mL of zinc acetate dehydrate added to the cultured medium. At the indicated time points, the medium was changed to 100 μL of MEM. Absorbance was measured for each well at the wavelength of 450 nm with an auto-microplate reader.

Apoptosis. M30 Apoptosense ELISA kit (Peviva, Bromma, Sweden) was used to determine the amount of caspase-cleaved cytokeratin 18 (cCK18). Huh7 cells (0.5×104) were seeded into a 96-well plate and cultured in 100 μL of MEM supplemented with 10% FBS. After 24 or 48 h had passed, the seeded cells were washed once with phosphate-buffered saline (PBS) and treated with 40 μg/mL of zinc acetate dehydrate added to the culture medium. The cells were lysed in poly-oxyethylene octylphenyl ether (NP-40) (FUJIFILM Wako Pure Chemical Corporation). The amounts of antigen in the controls and samples were calculated by interpolation from a standard curve.

Flow cytometric analysis. For the investigation of the mechanism underlying the inhibition of the tumor cells’ growth by zinc acetate dihydrate, we analyzed a cell-cycle profile after treatment with 40 μg/mL of zinc acetate dihydrate. The flow cytometric analysis was conducted using the Cell Cycle Phase Determinant kit (Cayman Chemical Co, Ann Arbor, MI, USA). Huh7 cells (1.0×106) were treated with 40 μg/mL of zinc acetate dehydrate or without zinc acetate for 24 or 48 h. The cells were then analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Indianapolis, IN, USA). The results were analyzed using Kaluza software (Beckman Coulter).

Gel electrophoresis and western blotting. The cell lysate was conducted according to the methods described in our previous reports (25). In brief, Huh7 cells were lysed using a protease inhibitor cocktail (Pro-Prep complete protease inhibitor mixture; iNtRON Biotechnology, Sungnam, Korea). Suspensions of lysed cells were centrifuged at 13,000 ×g at 4°C for 5 min. The supernatants containing soluble cellular proteins were then collected. Protein concentrations were determined using a NanoDrop 2000 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were electrophoresed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the proteins were transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies after blocking, and were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Perkin-Elmer, San Jose, CA, USA) on X-ray film.

As primary antibodies, the following antibodies were used: anti-β-actin monoclonal antibodies (used at 1 : 5,000; Sigma-Aldrich, St. Louis, MO, USA), cyclin D1 (used at 1 : 1,000; Thermo Fisher Scientific), cyclin E (used at 1 : 1,000; Thermo Fisher Scientific), cyclin-dependent kinase (Cdk) 6 (used at 1 : 1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Cdk4 (used at 1 : 1,000; Santa Cruz Biotechnology), and Cdk2 (used at 1 : 5,000; Santa Cruz Biotechnology). HRP-linked anti-mouse and anti-rabbit IgG (used at 1 : 2,000; GE Healthcare Life Science, Chalfont, UK) were selected as the secondary antibodies.

Antibody arrays for apoptosis-related proteins. A human apoptosis antibody array kit (R&D Systems, Minneapolis, MN, USA) was used to measure apoptosis-related proteins. In brief, apoptosis-related protein array membranes were blocked with 5% bovine serum albumin (BSA)/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h, and then incubated with 2 mL of lysate prepared from the above-described cells after normalization so that the amount of protein were equal. After three 10-min washes with TBS plus 0.1% v/v Tween-20 and two 10-min washes with TBS alone to remove unbound materials, the membranes were incubated with an HRP-conjugated anti-phosphotyrosine antibody for 2 h.
at room temperature. The unbound HRP antibody was washed out with TBS plus 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co, Waltham, MA, USA). The density of the immunoreactive band obtained from the apoptosis-related protein array was analyzed by densitometric scanning (Tlc Scanner, Shimizu Co, Ltd, Kyoto, Japan) (26).

**Analysis of the microRNA array.** We treated Huh7 cells with 40 μg/mL of zinc acetate dihydrate for 48 h and stored the cells in RNAprotect Reagent (Qiagen, Venlo, The Netherlands). As previously described in our studies (25, 26), total RNA was extracted from the cancer cell lines with the use of a miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The RNA samples typically exhibited A260/280 ratios between 1.9 and 2.1 using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After RNA measurements were conducted with an RNA 6000 Nano kit (Agilent Technologies), the samples were labeled using a miRCURYHy3/Hy5 Power Labeling kit and then hybridized on a human a miRNA Oligo chip (v.21.0; Toray Industries, Tokyo, Japan). The chips were scanned with a 3D-Gene Scanner 3000 (Toray Industries), and the results were analyzed using 3D-Gene extraction version 1.2 software (Toray Industries).

To determine the difference in miRNA expression between the zinc acetate-treated and control groups, we analyzed the raw data using GeneSpring GX 10.0 software (Agilent Technologies). Quantile normalization was conducted on the raw data that were above the background level. Differentially expressed miRNAs were determined by the Mann-Whitney U-test. Hierarchical clustering was performed using the further neighbor method with the absolute uncentered Pearson’s correlation coefficient as a metric. A heat map was produced with the relative expression intensity for each miRNA, in which the base-2 logarithm of the intensity was median-centered for each row (27).

**Statistical analyses.** Data were expressed as mean values±standard deviations. Two-way analysis of variances was used to compare the cell viability of treated and untreated HCC cell lines. Comparisons between the treatment and control groups were performed using the unpaired t-test. A p-value of <0.05 was considered as a statistically significant difference. Each experiment was repeated at least three times. Therefore, each result has reproducibility.

**RESULTS**

**Inhibitory effect of zinc acetate on the proliferation of human HCC cells**

The inhibitory effect of zinc acetate on the proliferation of the tumor cells was investigated with five human HCC cell lines: HepG2, Huh7, Hep3B, HLE and Alex. These HCC cells were cultured in the presence of 40, 50 and 60 μg/mL of or the absence of zinc acetate for 72 h. As shown in Fig. 1, the proliferation of human HCC cells was significantly inhibited at 72 h after the administration of zinc acetate in all five HCC cell lines. The inhibitory effect of zinc acetate was enhanced as the concentration of zinc acetate was increased, suggesting that zinc acetate exerts an inhibitory effect of
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Tumor proliferation in a dose-dependent manner. No effect of zinc acetate on cell cycle arrest

The flow cytometric analysis in Huh7 cells which was conducted to estimate the effect of zinc acetate on cell cycle progression in HCC cells revealed that the cell numbers treated with zinc acetate (40 μg/mL) were significantly lower in the G0/G1 phase and significantly higher in the G2/M and S phases compared to those without zinc acetate 24 h after the administration of zinc acetate (Fig. 2A, B). Similar results were obtained 48 h after the administration of zinc acetate (Fig. 2C, D). These results indicate that zinc acetate may accelerate cell cycle progression and may not contribute to cell cycle arrest in HCC cells.

We used western blotting to investigate the effects of zinc acetate on the expressions of the cell cycle-related proteins, including cyclin D1, cyclin E1, Cdk2, Cdk4 and Cdk6, in Huh7 cells. As shown in Fig. 3, the bands corresponding to Cdk4 and Cdk6, which are catalytic subunits of cyclin D1 and associated with the G0 to G1 transition, were more intense 24 h after treatment with zinc acetate (40 μg/mL) compared to the bands without zinc acetate. The bands for cyclin E1, which is the key cyclin for the G1/S transition, and Cdk2 (catalytic subunit of cyclin E1) were also stronger than those without zinc acetate. However, the intensity of the bands for cyclin D1 was approximately equivalent between the

tumor proliferation in a dose-dependent manner.

no effect of zinc acetate on cell cycle arrest

Fig. 2. Alteration of cell cycle progression in Huh7 cells by the administration of zinc acetate. A flow cytometric analysis of the cell cycle in Huh7 cells at 24 and 48 h after the administration of zinc acetate (40 μg/mL) was conducted. *p<0.05, **p<0.01 vs. untreated Huh7 cells. Zn (-): untreated, Zn (+): treated with zinc acetate.

Fig. 3. Changes in the cell cycle-related proteins by the administration of zinc acetate. A western blot analysis of cell cycle-related proteins at 24 h after the administration of zinc acetate was performed. Zn (-): untreated, Zn (+): treated with zinc acetate.
zinc acetate group and the control group. 

**Induction of apoptosis of Huh7 cells by the administration of zinc acetate**

To examine the effect of zinc acetate dihydrate on the apoptosis of Huh7 cells, we used the M30 apotosense method which determines the cCK18 levels in apoptotic cells. At 24 h after the zinc acetate dihydrate (40 μg/mL) administration, the supernatant cCK18 levels were significantly higher in the group treated with zinc acetate dihydrate compared to the control group (159.1 ± 38.2 U/L vs. 111.6 ± 26.0 U/L, p<0.05) and the significant difference remained at 48 h after the administration (301.7 ± 73.6 U/L vs. 192.0 ± 38.1 U/L, p<0.05) (Fig. 4), indicating that the administration of zinc acetate resulted in apoptosis of the HCC cells.

**Enhancement of the heme oxygenase-1 expression in Huh7 cells by administration of zinc acetate**

We performed the antibody array for apoptosis-related protein in order to identify which protein was involved in the process of Huh7 cells' apoptosis following the administration of zinc acetate among 35 apoptosis-related proteins. The expression of heme oxygenase-1 (HO-1) in Huh7 cells treated with zinc acetate (40 μg/mL) was enhanced, compared to that in Huh7 cells without zinc acetate (Fig. 5).

**Effects of zinc acetate dihydrate on the miRNA expression in Huh7 cells**

The miRNA profiles obtained 48 h after the treatment with zinc acetate dihydrate (40 μg/mL) in vitro were compared with the profiles of control in order to identify miRNAs that were upregulated or downregulated in response to the treatment. The unsupervised hierarchical clustering analysis indicated that the group treated with zinc acetate dihydrate was clustered sepa-
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readily from the control group (Fig. 6). Table 1 lists the miRNAs whose fold changes were $>1.5$ or $<1/1.5$, with a $p$-value$<0.0001$ by the treatment with zinc acetate dihydrate. As shown in the table, 13 upregulated miRNAs and six downregulated miRNAs were identified.

### DISCUSSION

Our findings clarified the antitumor effect of zinc acetate on HCC cells via the initiation of cell apoptosis. To the best of our knowledge, this is the first report to exert a beneficial effect of zinc acetate on HCC cells in a dose dependent manner. Other studies have revealed that the administration of zinc sulfate caused apoptosis in prostate cancer, breast cancer and HCC cells (16–18, 20). It was suggested that the treatment of prostate cancer with zinc sulfate might increase the intracellular zinc levels, resulting in the initiation of cancer cells' apoptosis (16).

Our present results also revealed that the treatment with zinc acetate increased the HO-1 expression in HCC cells. Likewise, the administration of zinc chloride increases HO-1 expression in human neuroblastoma cells (28). HO-1, also known heat shock protein 32, has a variety of functions, including anti-apoptosis and anti-oxidant properties (29). HO-1 is expressed at low level under basal condition, but its expression was increased in various types of cancer cells. Indeed, HO-1 modulated the apoptosis of renal cancer cells and consequently promoted their survival (30). Another study
revealed that low grade HO-1 expression promoted the apoptosis of gastric cancer cells (31).

In contrast, there is an interesting article noting that the upregulation of HO-1 resulted in remarkable apoptosis of colorectal cancer cells (32). The discrepancy in findings may be derived from the severity of HO-1 up-regulation and/or the types of cancer cells. We could not conclude whether or not HO-1 has an anti-apoptotic effect on HCC cells in this study. We speculate that upregulated HO-1 expression might result in the apoptosis of the HCC cells, because the expression of cleaved caspase-3, which plays a crucial role of apoptosis, was not upregulated in the HCC cells. Unfortunately, the reason why cleaved caspase-3 expression was not upregulated remains uncertain. However, an additional administration of the HO-1 inhibitor (zinc protoporphyrin-IX) would demonstrate the contribution of HO-1 to the apoptosis of cancer cells. Further examinations are thus necessary to clarify the function of HO-1 in the HCC cells treated the zinc acetate.

As mentioned above, HO-1 possesses another function, i.e., anti-oxidant action (29). Accordingly, an upregulation of HO-1 in HCC cells may alleviate oxidative stress in those cells, leading to an antitumor effect regardless of the cells’ apoptosis.

Our present study demonstrated that zinc acetate did not affect the cell cycle progression of the HCC cells, although another zinc compound (zinc sulfate) caused G1 and G2/M arrest in various type of cancer cells (14, 17). Therefore, a combination treatment of zinc acetate plus a cytotoxic agent that causes cell cycle arrest would substantially enhance the antitumor effect in HCC cells. Further studies should be conducted to confirm the efficacy of this potential combination treatment in those cancer cells.

Zinc also increases cancer cells’ chemosensitivity to a cytotoxic agent such as paclitaxel (20). The combination treatment of the cytotoxic agent with a zinc compound may thus be a potential strategy for HCC. Other possible mechanisms by which zinc administration exerts antitumor effects, have been proposed, including a mechanism involving the genomic stability of cancer cells (33, 34) and the downregulation of hypoxia-inducible factor-α (HIF-1α) in tumor cells (35).

One of the miRNAs upregulated by zinc acetate in this study, miR-143-3p, was directly bound to a novel lncRNA, called LINC00908, and its expression was negatively correlated with the LINC00908 expression in colorectal cancer tissue (36). The inhibition of LINC00908 resulted in the suppression of the colorectal cancer cells’ growth. These results indicated that upregulation of miR-143-3p was likely to trigger apoptosis of HCC cells by the treatment with zinc acetate.

On the other hand, miR-21-5p, another miRNA upregulated by the treatment with zinc acetate in the present study, targeted kruppel-like factor 6 (KLF6), which is a member of the family of sp1/KLF transcription factor, and its knockdown prominently elevated the KLF6 level in HCC cells. The increased expression of KLF6 in HCC cells induced the apoptosis of HCC cells and suppressed the cells’ viability, migration and invasion, indicating that a downregulation of miR-21-5p exerted antitumor effects in the HCC cells (37). These results are absolutely opposite to our present observation that upregulating miR-21-5p resulted in the apoptosis of HCC cells via the administration of zinc acetate. The reasons for the discrepancy in results require further investigation.

Zinc deficiency is one of the risk factors for cancer development, and it was reported to result in the progression of HCC (38, 39). It is of interest that Fong et al. described a close relationship between the alteration of the miRNA profile and the development of esophageal cancer in zinc-deficiency status, and they identified several miRNAs were identified as the zinc-deficiency-associated miRNAs (40). miR-31 was one of upregulated miRNAs induced by zinc deficiency (41). The miRNA expression was accompanied by downregulation of a tumor suppressor target PPP2R2A. The authors speculated that downregulation of such a tumor suppressor target might be likely to cause cell proliferation and inflammation under the condition of zinc deficiency.

There are several limitations in this study. First, it was an in vitro study only. Therefore, the antitumor effect of zinc acetate should be examined in vivo study. Second, other putative mechanisms by which zinc acetate induces antitumor effects, including the inhibition of angiogenesis and tumor cells’ invasion, were not fully investigated. Third, direct evidence that any miRNAs are involved in the apoptosis of HCC cells was not obtained in this study herein.

In conclusion, the administration of zinc acetate resulted in remarkable antitumor effect on five HCC cell lines in a dose-dependent manner. The antitumor effect of zinc acetate appears to be responsible for the apoptosis of the cancer cells, but it did not affect the cell cycle progression. The HCC cells’ apoptosis may be associated with the upregulation of HO-1 in those cells and the alteration of the miRNA profile. Zinc acetate may thus be a promising agent as an adjunct to a conventional systemic chemotherapy for HCC.

**Authorship**

TM designed the experimental protocol. RH and MY conducted the experiments. RH wrote the manuscript. KF, SM, JT, and AM provided technical supports. TH and TM edited the manuscript.

**Disclosure of state of COI**

All authors have no conflicts of interest to declare.

**Funding**

This research received no external funding.

**Data availability**

The data presented in this article will be provided on request based on reasonable queries.
Antitumor Effects of Zinc Acetate in HCC


