

# Involvement of NF- $\kappa$ B activation in the cisplatin resistance of human epidermoid carcinoma KCP-4 cells

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**Abstract.** *cis*-Diamminedichloroplatinum II (cisplatin) is one of the most potent antitumor agents for the treatment of various types of cancer. In spite of its therapeutic usefulness, the intrinsic resistance acquired under continuous treatment limits its benefit in cancer therapy. KCP-4, a cisplatin-resistant cell line, was derived from human epidermoid carcinoma KB-3-1 cells. Since the accumulation of cisplatin in KCP-4 cells is markedly reduced by the presence of an efflux pump, this pump is thought to be related to cisplatin resistance of the KCP-4 cells. However, given that KCP-4 cells are tremendously resistant to cisplatin compared with KB-3-1 cells, it is possible that another mechanism exists. The aim of this study was to investigate whether the activation of nuclear factor-kappa B (NF- $\kappa$ B) contributes to the cisplatin resistance of KCP-4 cells. We used the level of translocated NF- $\kappa$ B into the nucleus, determined by immunoblot analysis, as the indicator of NF- $\kappa$ B activation. The activation level of NF- $\kappa$ B was higher in KCP-4 cells than in KB-3-1 cells. KCP-4 cells were treated with a combination of cisplatin and curcumin, an inhibitor of NF- $\kappa$ B activation, and the cell viabilities were subsequently determined by the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. In the presence of 10  $\mu$ mol/l curcumin, we found that the sensitivity of KCP-4 cells to 100 and 300  $\mu$ mol/l cisplatin was augmented. Additionally, curcumin reduced the

activation levels of NF- $\kappa$ B in KCP-4 cells, and suppressed the expression levels of Bcl-2, Bcl-xL and survivin, which are apoptosis-related proteins regulated by NF- $\kappa$ B. Our results suggest that the high cisplatin resistance of KCP-4 cells compared with KB-3-1 cells results from multiple mechanisms other than increased cisplatin efflux, including the activation of NF- $\kappa$ B.

## Introduction

*cis*-Diamminedichloroplatinum II (cisplatin) is one of the most potent antitumor agents in cancer chemotherapy (1). It is generally accepted that the cytotoxic activity of cisplatin results from its interactions with DNA, including the inhibition of DNA replication, DNA repair, disturbance of the cell cycle, and beneficial process of apoptosis in cancer therapy (2-8). Although cisplatin is highly effective in the treatment of various cancers (head and neck, testicular, ovarian, bladder, germ cell and small-cell lung cancers), intrinsic or acquired resistance sometimes reduces its efficacy (9,10). The mechanisms of resistance include declined accumulation of cisplatin due to decreased uptake and/or increased efflux, accelerated DNA repair, inactivation of the cisplatin by glutathione, altered apoptosis-related signals and the activation of signaling pathways (10,11).

Many proteins are involved in cisplatin resistance that is mediated by altered apoptosis-related signals and the activation of signaling pathways. It is reported that some forms of resistance are caused by the altered expression of proteins such as Bcl-2 family members (12) and the activation of signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway (11,13). One of the factors that can influence the expression of Bcl-2 family members or the MAPK signaling pathway is nuclear factor- $\kappa$ B (NF- $\kappa$ B) (14-17). Aberrant NF- $\kappa$ B regulation has been observed in many cancers and has been shown to make some cancer cells resistant to antitumor agents (18). Furthermore, it is reported that NF- $\kappa$ B inhibitors augment cisplatin's antitumor capabilities against some cisplatin-resistant cell lines (19,20).

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We have investigated the resistance mechanisms of cisplatin-resistant KCP-4 cells derived from the human epidermoid carcinoma cell line KB-3-1 (26,27). In KCP-4 cells, the accumulation of cisplatin was markedly reduced compared with the parent KB-3-1 cells. The time-dependent cisplatin accumulation in KCP-4 cells rapidly decreased, after an initial, transient increase, in response to the addition of cisplatin to culture medium. This accumulation was enhanced by 2,4-dinitrophenol, an inhibitor of the phosphorylation of ADP to ATP. Therefore, it has been proposed that the ATP-dependent cisplatin efflux system exists in KCP-4 cells (26-28).

To investigate the involvement of NF- $\kappa$ B in the cisplatin resistance of KCP-4 cells, we drew on the properties of the compound curcumin, a known inhibitor of NF- $\kappa$ B. Curcumin is a yellow pigment found in the spice turmeric, derived from the rhizome of the plant *Curcuma longa*. Curcumin has shown strong anti-inflammatory, antioxidant, and anticancer properties (21). It has also been shown to suppress the activation of NF- $\kappa$ B, resulting in the reduction of the expression of various NF- $\kappa$ B-regulated proteins, such as Bcl-2 and Bcl-xL (22,23). It is reported that curcumin sensitizes some cisplatin-resistant cells to cisplatin by changing the NF- $\kappa$ B signaling pathway (24,25). In this study, we have investigated the involvement of NF- $\kappa$ B activation in KCP-4 cisplatin resistance. We demonstrate that curcumin enhances the sensitivity of KCP-4 to cisplatin, accompanied by the reduction of activated NF- $\kappa$ B without changing the intracellular accumulation of cisplatin. This suggests that altered apoptosis-related signals and activation of signaling pathways are also involved in the cisplatin resistance of KCP-4.

## Materials and methods

**Cell culture.** Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA). Human epidermoid carcinoma KB-3-1 and KCP-4 cells were cultured in DMEM containing 10% fetal bovine serum and 100 U/ml of penicillin (Invitrogen) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

**MTT assay.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and curcumin were purchased from Wako (Osaka, Japan). Cisplatin was purchased from Sigma (St. Louis, MO, USA). The MTT colorimetric assay was used to determine the relative sensitivity of cell lines to cisplatin and/or curcumin. KB-3-1 and KCP-4 cells were seeded in each well of 96-well plates at  $3 \times 10^3$  and  $1 \times 10^4$  cells/well, respectively, and cultured for 24 h. Cells were treated with cisplatin for 48 h. In the combination treatment with curcumin, cells were precultured with curcumin for 24 h. In a dose-response analysis for curcumin, cells were cultured with curcumin for 72 h. After the individual culture, 50  $\mu$ l of MTT (1 mg/ml in PBS) was added to each well, and the cells were cultured for an additional 4 h. The resultant formazan was dissolved with 100  $\mu$ l of dimethyl sulfoxide after aspiration of the culture medium. Plates were placed on a plate shaker for 5 min, and then the optical density was read immediately at 595 nm using a model DTX 880 microplate reader (Beckman Coulter, Fullerton, CA, USA).

**Cisplatin accumulation.** To measure intracellular accumulation of cisplatin, KB-3-1 and KCP-4 cells were cultured for 24 h in DMEM and then cultured with cisplatin (300  $\mu$ mol/l) for 2 h at 37°C. Cells were washed 3 times with cold PBS and immediately harvested. The harvested cells were further washed with cold PBS, and cell numbers were counted with a hemocytometer before the aspiration of PBS. Cell pellets were lysed in nitric acid, and the concentrations of platinum were determined by inductive coupled plasma spectrometry (ICP) using a Model Optima 4000 apparatus (Perkin-Elmer, Tokyo, Japan).

**Preparation of nuclear extracts.** Nuclear extracts were prepared using a Nuclear/Cytosol Fractionation kit (Wako) according to the manufacturer's instructions. Briefly, collected cells were resuspended in cytosol extraction buffer, followed by vigorous vortex, and centrifuged at 16,000  $\times$  g for 5 min at 4°C. The supernatant fractions were transferred into another tube as cytoplasmic extracts. The pellets were resuspended in nuclear extraction buffer and vortexed vigorously 4 times for 15 sec every 10 min. The samples were then centrifuged at 16,000  $\times$  g for 10 min at 4°C. The supernatants were used as nuclear extracts. NF- $\kappa$ B present in the nuclear extracts was detected by immunoblot analysis, as described below.

**Immunoblot analysis.** Complete EDTA-free was purchased from Roche Diagnostics (Indianapolis, IN, USA), and sodium dodecyl sulfate (SDS) was from Sigma. Antibodies against NF- $\kappa$ B p65, I- $\kappa$ B $\alpha$ , Bcl-xL and Bcl-2, along with HRP-conjugated donkey anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated goat anti-mouse and -rabbit antibodies were purchased from Nacalai Tesque (Kyoto, Japan). All other reagents for immunoblot analysis were purchased from Wako. Whole-cell lysates were prepared by lysing with detergent buffer [10 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA, 150 mmol/l NaCl, 1% (v/v) Triton X-100, 10% glycerol, complete EDTA-free, and 1 mmol/l benzylsulfonyl fluoride]. Insoluble fractions were removed by centrifugation at 16,000  $\times$  g for 10 min at 4°C. Whole-cell lysates and nuclear extracts were boiled in a quarter volume of sample buffer (125 mmol/l Tris-HCl, pH 7.5, 25% glycerol, 5% SDS, 0.2% bromophenol blue and 25% 2-mercaptoethanol). Proteins in these samples were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated for 1 h in Tris-buffered saline (TBS) solution containing 5% non-fat milk as blocking buffer, and then treated overnight with primary antibodies in blocking buffer at 4°C. The membrane was washed in TBS and then incubated with 1:3,000 diluted antibody (anti-mouse, -rabbit, or -goat IgG) labeled with HRP in blocking buffer at room temperature for 1 h. It was then washed again in TBS. Antibody binding was visualized with ECL Plus Western Blotting Detection system (GE Healthcare Bio-Sciences, Buckingham, UK).

**Statistical analysis.** Differences between groups were assessed by one-way ANOVA followed by Tukey's test for multiple comparisons. Data are presented as means  $\pm$  SD. Differences were considered significant at  $P < 0.05$ .

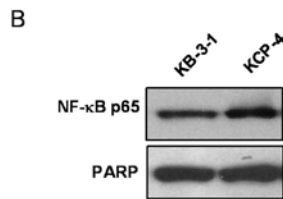
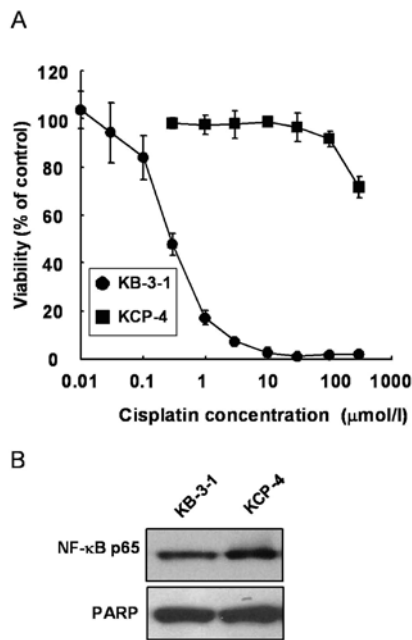


Figure 1. Comparison of the sensitivity to cisplatin and intranuclear NF- $\kappa$ B expression in KB-3-1 and KCP-4 cells. (A) Sensitivity of KB-3-1 and KCP-4 cells to cisplatin. Viability, expressed as a percentage of the control, was measured 48 h after addition of cisplatin to the culture medium. The data are expressed as mean  $\pm$  SD of 6 independent experiments. (B) Expression levels of nuclear NF- $\kappa$ B in nuclear fraction of KB-3-1 and KCP-4 cells.

## Results

**Characteristics of KB-3-1 and KCP-4 cells.** To determine the degree of resistance of KCP-4 to cisplatin, the sensitivities of KB-3-1 and KCP-4 to cisplatin were determined by the MTT assay. As shown in Fig. 1A, KCP-4 cells were much more resistant to cisplatin than the parental KB-3-1 cells.  $EC_{50}$  values of cisplatin for KB-3-1 and KCP-4 cells were about 0.3  $\mu$ mol/l and >300  $\mu$ mol/l, respectively. Next, the levels of NF- $\kappa$ B translocated into nucleus were used as an indication of NF- $\kappa$ B activation. These levels were measured by immunoblot analysis, revealing that the activation level of NF- $\kappa$ B was higher in KCP-4 than in KB-3-1 cells (Fig. 1B).

**Effects of curcumin on the sensitivity of KCP-4 cells to cisplatin and the accumulation of platinum in KCP-4 cells.** First, a dose-response analysis was performed using the MTT assay to determine the level of curcumin administration in KCP-4 cells at which there was no observed adverse effect. As shown in Fig. 2A, a dose of <10  $\mu$ mol/l curcumin had no significant effect on the viability of KCP-4. Therefore, subsequent sensitivity studies were carried out using 3 and 10  $\mu$ mol/l curcumin. Next, we investigated if curcumin can enhance the sensitivity of KCP-4 cells to cisplatin. The viabilities of KCP-4 cells treated with 30, 100 and 300  $\mu$ mol/l cisplatin were 96.6 $\pm$ 5.9, 91.6 $\pm$ 3.3 and 71.5 $\pm$ 4.4%, respectively, of that of the control. When cells were treated with a combination of 10  $\mu$ mol/l curcumin and 100 or 300  $\mu$ mol/l cisplatin, the viability of KCP-4 cells was significantly decreased (Fig. 2B,  $P<0.05$ ). The viabilities were 72.8 $\pm$ 7.7 and 19.9 $\pm$ 3.9% for 100 and 300  $\mu$ mol/l cisplatin, respectively. In concentrations of cisplatin <30  $\mu$ mol/l, the sensitivity was not enhanced by the

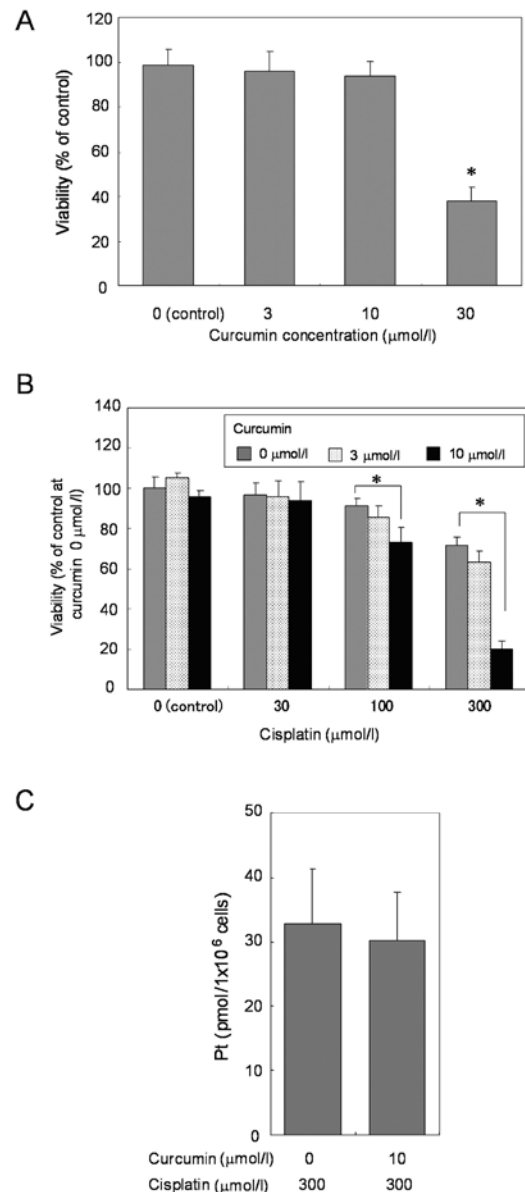


Figure 2. Effects of combined curcumin and cisplatin treatment on the viability of KCP-4 cells. (A) Sensitivity of KCP-4 cells to curcumin. Viability, expressed as a percentage of the control, was measured 72 h after addition of curcumin to the culture medium. The data are expressed as mean  $\pm$  SD of 6 independent experiments. \* $P<0.05$  compared with the control. (B) Sensitivity of KCP-4 cells to curcumin and cisplatin combination. KCP-4 cells plated in culture dishes were pretreated with curcumin for 24 h, and then cisplatin was added to each well. Viability was measured by the MTT assay 48 h after cisplatin treatment. The data are expressed as mean  $\pm$  SD of 6 independent experiments. \* $P<0.05$  compared to the viability of KCP-4 at 0  $\mu$ mol/l curcumin. (C) Accumulation of platinum in KCP-4 cells with or without curcumin treatment. KCP-4 cells were cultured with 300  $\mu$ mol/l cisplatin in the presence or absence of curcumin. Intracellular concentrations of platinum were determined by inductive coupled plasma spectroscopy as described in Materials and methods.

combined treatment of curcumin and cisplatin (Fig. 2B). The intracellular accumulation of platinum in curcumin-treated KCP-4 cells was not significantly different from that in control KCP-4 cells (Fig. 2C).

**Effects of curcumin on the nuclear translocation of NF- $\kappa$ B in KCP-4 cells.** The expression levels of nuclear NF- $\kappa$ B in KCP-4

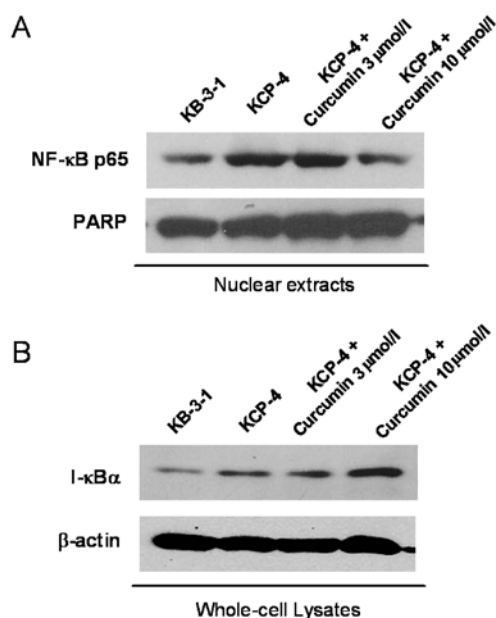


Figure 3. Effects of curcumin on the activation of NF- $\kappa$ B in KCP-4 cells. KCP-4 cells were treated with 3 or 10  $\mu$ mol/l curcumin for 72 h. (A) Expression of NF- $\kappa$ B in the intranuclear fraction of KB-3-1 and KCP-4 with or without curcumin. (B) Expression of I- $\kappa$ B $\alpha$  in whole-cell lysates of KB-3-1 and KCP-4, with or without curcumin.

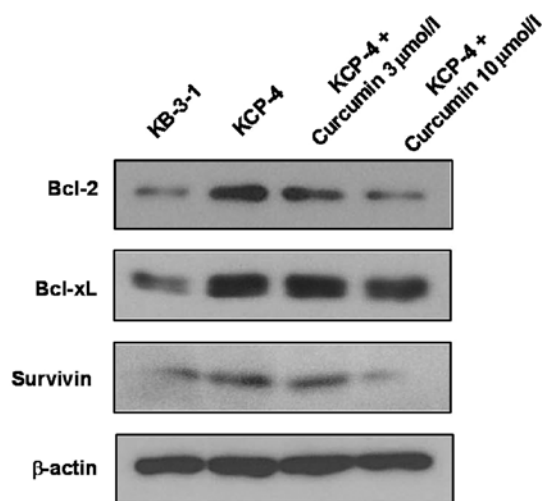


Figure 4. Effects of curcumin on the expression of apoptosis-related proteins in KCP-4 cells. KCP-4 cells were treated with 3 or 10  $\mu$ mol/l curcumin for 72 h. Expressions of Bcl-2, Bcl-xL and survivin in whole-cell lysates of KB-3-1 and KCP-4, with or without curcumin.

treated with curcumin for 72 h was investigated by immunoblot analysis. As shown in Fig. 3A, expression was higher in KCP-4 than in KB-3-1. Curcumin reduced the level of nuclear NF- $\kappa$ B in KCP-4 cells. Additionally, the level of I- $\kappa$ B in KCP-4 cells was increased by curcumin treatment (Fig. 3B).

*Effects of curcumin on the expression of apoptosis-related proteins in KB-3-1 and KCP-4 cells.* Effects of curcumin on the expression levels of Bcl-2, Bcl-xL and survivin were investigated by immunoblot analysis. As shown in Fig. 4, the expression levels of Bcl-2, Bcl-xL and survivin in KCP-4

were higher than in KB-3-1 cells. Curcumin treatment for 72 h reduced the expression of Bcl-2, Bcl-xL and survivin in KCP-4 cells.

## Discussion

KCP-4 is a cisplatin-resistant cell line derived from the human epidermoid carcinoma cell line KB-3-1. Previous studies have shown that the accumulation of cisplatin in KCP-4 is markedly decreased by any efflux pump. However, because the resistance of KCP-4 cells to cisplatin is approximately 1,000-fold greater than that of KB-3-1 cells, the existence of another resistant mechanism has been proposed. Here, we focused on the relationship between NF- $\kappa$ B activation and cisplatin resistance of KCP-4 cells. It is known that NF- $\kappa$ B is sometimes related to the resistance to many chemotherapeutic agents in various cancer cells. The present study demonstrates that curcumin, an inhibitor of NF- $\kappa$ B, enhances the sensitivity of KCP-4 to cisplatin without changing intracellular cisplatin accumulation. Therefore, curcumin may affect an intracellular signalling pathway, without having any effects on the cisplatin efflux system of KCP-4. The activation of NF- $\kappa$ B was enhanced in KCP-4 cells compared with KB-3-1, but was reduced by curcumin treatment, suggesting that enhanced NF- $\kappa$ B may be related to the resistance of KCP-4. The expression levels of Bcl-2, Bcl-xL and survivin, which are regulated by NF- $\kappa$ B (23,29), were enhanced in KCP-4 compared with KB-3-1 and reduced by curcumin treatment. This suggests the involvement of NF- $\kappa$ B-regulated apoptotic proteins in the resistance mechanism of KCP-4.

NF- $\kappa$ B molecules are transcription factors composed of Rel family molecules, including p65(RelA), p50(NF- $\kappa$ B1), NF- $\kappa$ B2(p52), cRel and RelB, which associate with one another in various heterodimeric and homodimeric combinations (30). The heterodimer of p65 and p50, known as classical NF- $\kappa$ B, is present in the cytoplasm in an inactive form bound to the inhibitory protein I- $\kappa$ B. Signal activation by phosphorylation and degradation of I- $\kappa$ Bs enables NF- $\kappa$ B to translocate into the nucleus and bind to the  $\kappa$ B site, regulating the expression of many genes. Some reports describe a relationship between resistance to cisplatin and NF- $\kappa$ B activation. In one such report, radiation-induced cisplatin resistance in HeLa/B cells appeared to be mediated by enhanced activation levels of NF- $\kappa$ B (31). In another report, cisplatin-sensitive ovarian cancer A2780 cells did not demonstrate constitutive activation of NF- $\kappa$ B. However, cisplatin-resistant ovarian cancer CAOV-3 cells demonstrated constitutive activation of NF- $\kappa$ B. Furthermore, BAY 11-7085, an inhibitor of I- $\kappa$ B $\alpha$  phosphorylation, decreased NF- $\kappa$ B activation and enhanced the sensitivity of CAOV-3 cells to cisplatin (19). Constitutive NF- $\kappa$ B activation makes cancer cells resistant to various chemotherapeutic agents as well as cisplatin (32).

NF- $\kappa$ B is activated by many chemotherapeutic agents, including cisplatin, doxorubicin, etoposide and paclitaxel (32). The KCP-4 cell line was established under conditions of low cisplatin concentration (27), so that NF- $\kappa$ B in KCP-4 cells might be activated by the stimulatory capacity of cisplatin. In clinical practice, the resistance to cisplatin is acquired under continuous treatment with cisplatin, and thus, the activation of

NF- $\kappa$ B by the stimulation of cisplatin might contribute to the acquisition of cisplatin resistance. Therefore, it is expected that inhibition of NF- $\kappa$ B activation would prevent the acquisition of cisplatin resistance.

Curcumin can be used to study this inhibition. This natural component of the spice turmeric is derived from the rhizome of the East Indian plant *C. longa* (21). It has been extensively used in Ayurvedic medicine for centuries, as it has a variety of therapeutic properties, including antioxidant, analgesic, anti-inflammatory and antiseptic activities (21). It has been shown that curcumin prevents tumor initiation, proliferation, and metastasis in breast, colon, oral, ovarian, and many other human cancers (33). It also enhances the sensitivity of various cancer cells to cisplatin by suppressing the activation of NF- $\kappa$ B, thereby inducing downregulation of target genes such as Bcl-2 and Bcl-xL (25,34,35). In the treatment of head and neck tumors, there is a significant interest in using curcumin as an adjuvant agent in combination with standard cisplatin chemotherapy (21).

The present study shows that curcumin enhances the sensitivity of KCP-4 cells to cisplatin, thus suggesting the utility of combining cisplatin and curcumin in cancer treatment. It is expected that additional research will be conducted into the practicality of such combined treatment due to the frequent use of cisplatin in the treatment of head and neck cancers. However, curcumin has poor bioavailability and is water insoluble, and so, its distribution in the body is limited (35). Wang *et al* reported the use of liposome-encapsulated curcumin for intravenous administration and confirmed the presence of curcumin in the circulating blood and liver in a bioavailability study (36). Additionally, significant growth inhibition has been demonstrated in xenograft tumors treated with a combination of liposomal curcumin and cisplatin (35,36). It is hoped that other drug delivery systems will be developed to yield superior bioavailability and therapeutic results.

There are many mechanisms associated with cisplatin resistance, including decreased accumulation due to the decreased uptake and/or increased efflux of cisplatin, accelerated DNA repair, inactivation of cisplatin by glutathione, altered apoptosis-related signals, and the activation of signaling pathways (10,11). It has already been reported that an ATP-dependent cisplatin efflux pump, as yet unidentified, exists in KCP-4 cells and that the high expression of the pump contributes to the resistance of KCP-4 cells to cisplatin (26-28). In the present study, curcumin had no effect on the accumulation of cisplatin in KCP-4 cells, suggesting that the activation of NF- $\kappa$ B and the cisplatin efflux system are independent mechanisms of cisplatin resistance. It is thought that the sensitivity of KCP-4 cells to cisplatin at low concentrations was not affected by curcumin because the accumulation of cisplatin was kept at low level by the cisplatin efflux system.

In conclusion, our study shows that one of the mechanisms for cisplatin resistance in KCP-4 cells is the activation of NF- $\kappa$ B and that curcumin suppresses both NF- $\kappa$ B activation and the expression of some NF- $\kappa$ B-regulated apoptosis-related proteins. It is thought that KCP-4 cells are highly resistant to cisplatin due to multiple changes in the NF- $\kappa$ B-activation pathway and increased cisplatin efflux. Further study is necessary to identify the cisplatin efflux pump that is being overexpressed in KCP-4 cells.

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