Unconjugated Bilirubin Is a Novel Prognostic Biomarker for Nasopharyngeal Carcinoma and Inhibits Its Metastasis via Antioxidation Activity

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Abstract

Distant metastasis is the most common cause of treatment failure and mortality in nasopharyngeal carcinoma (NPC) patients. Thus, it is important to understand the mechanism of NPC metastasis and identify reliable prognostic factors. In this study, we investigated the prognostic value of unconjugated bilirubin (UCB), which was previously considered a byproduct of heme catabolism, in NPC patients and examined the effects of UCB on NPC metastasis. The receiver operating characteristic analysis–generated UCB cutoff point for DMFS was 9.7 μmol/L. We found that higher UCB levels were significantly associated with favorable distant metastasis-free survival (DMFS, 93.3% vs. 84.2%, P < 0.001) in NPC patients and was an independent predictor for DMFS (HR, 0.416; 95% confidence interval, 0.280–0.618; P < 0.001). We next found that UCB treatment impaired the invasion capability of NPC cells and potently inhibited lung metastasis of NPC cells in nude mice. Further investigation showed that UCB inhibited reactive oxygen species production, which is involved in the repression of ERK1/2 activation and matrix metalloproteinase-2 (MMP-2) expression. Moreover, lower levels of ERK1/2 phosphorylation and MMP-2 expression were observed in the NPC lung metastases of nude mice administered UCB. Taken together, our results indicate that UCB is a significantly favorable factor for DMFS in NPC patients and may play an important role in NPC chemoprevention.

Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy in South China, North Africa, and Alaska (1–3). In South China, the annual incidence of NPC was reported 25 to 30 per 100,000 individuals (1). NPC is highly malignant and often invades adjacent regions and metastasizes to regional lymph nodes or distant organs. Although early-stage NPC patients are sensitive to radiotherapy and chemotherapy, treatment failure is common in late-stage patients due to distant metastases, which are the key contributors to NPC mortality and highlight the need for both further understanding of NPC metastasis and novel drugs to treat it (4, 5).

Bilirubin, a major product of heme catabolism, is generated by the hemeoxygenase-mediated oxidation of heme during the production of biliverdin. Unconjugated bilirubin (UCB) is produced by biliverdin reductase–catalyzed reduction. UCB is cleared from the circulation by the liver, where it is conjugated with glucuronic acid by the enzyme bilirubin UDP-glucuronosyl transferase (UGT1A1) to form conjugated bilirubin, which enters the small intestine as a component of bile. Until the discovery of its antioxidant effects, bilirubin was considered a waste product of the body (6). Currently, an increasing number of studies have suggested the potential protective effects of bilirubin against oxidative stress–related diseases, including stroke, carotid artery atherosclerosis, coronary heart disease, respiratory disease, and cancer (7–17). However, few studies have investigated the effects of bilirubin on tumor metastasis.

Free radicals and reactive molecules containing oxygen are known as reactive oxygen species (ROS) and induce oxidative stress in cells (18). Extensive research has revealed that ROS and oxidative stress may mediate many behaviors of cancers, including transformation, survival, proliferation, chemoresistance, radioresistance, angiogenesis, and metastasis (19, 20). Thus, ROS inhibition is important in cancer chemoprevention. Oxidative stress can activate many molecules, such as Ras, PI3K/Akt, ERK1/2, p38 MAPK, and JNK1/2, and inactivate the phosphatases that regulate these proteins (21). Activation of these molecules often...
leads to upregulation of the activity and expression of matrix metalloproteinase (MMP) proteins, which are associated with the invasion and metastasis of malignant tumors of various histogenetic origins (22, 23).

In this study, we used a large-scale, retrospective cohort study method and in vitro and in vivo NPC metastasis models to examine the association between bilirubin levels and NPC metastasis. We found that UCB is a favorable prognosis biomarker for DMFS in NPC patients and that it can inhibit the metastasis of NPC cells both in vitro and in vivo by repressing ERK1/2 activation and MMP-2 expression via its antioxidation activity. Our results indicate the first time that UCB can inhibit the metastasis of NPC and may play an important role in chemoprevention.

Materials and Methods

Patients’ recruitment and data collection
In total, 1,327 histologically diagnosed nonmetastatic NPC patients at the Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China) between January 2005 and December 2007 were included in the current study. Fifteen patients with unknown personal information, such as gender, age, and TNM classification, and 50 patients with liver dysfunction were excluded. The clinical characteristics are summarized in Supplementary Table S1. All patients were staged according to the 6th edition of the Union Internationale Contra Cancer/american Joint Committee on Cancer TNM classification system. Informed consent was obtained, and the study was approved by the institutional ethical board at the SYSUCC. Baseline bilirubin levels were obtained before treatment and determined by an automated immunoturbidimetric analyzer 7600-020 (Hitachi, High-Technologies).

Cell lines
The NPC cell line 5-8F and two single-cell clones derived from the NPC cell line CNE-2, CNE-2-S18 (S18) and CNE-2-S26 (S26), were kindly provided by Dr. Chao-Nan Qian, Sun Yat-sen University Cancer Center, China (24). The other NPC cell line, HONE-1, was also obtained from the SYSUCC. All cell lines were thawed from early-passage stocks and passaged for less than 6 months. Each cell line was authenticated in March 2013 and was periodically monitored for mycoplasma using Hoechst staining. Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (BSA) was from Merck Millipore. When UCB was used, BSA was added to the culture medium at a UCB to BSA ratio of 1. The MEK1/2 inhibitor U0126 and reactive oxygen species assay kits were purchased from Beyotime.

Migration and invasion assays
Migration and invasion assays were performed as previously described (25). S18 cells (2 × 10^4 cells/well for migration assays) and HONE-1 cells (5 × 10^4 cells/well for migration assays and 8 × 10^4 cells/well for invasion assays) were plated on the inserts and cultured in the upper chambers at 37°C for 24 hours. The number of migrated and invaded cells in five random optical fields (>100 magnification) from triplicate filters was counted and averaged.

In vivo metastasis and antitumor assay
All in vivo experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. For the metastasis assay, S18 cells were injected into the tail veins of BALB/c nude mice (1 × 10^6 cells/0.1 ml DMEM). Daily treatment by intraperitoneal injection of 20 mg/kg or 30 mg/kg body weight UCB or DMSO began 3 days before cells were injected and lasted 7 weeks. Then, the animals were euthanized and the lungs were collected, weighed and separated into two parts. Total RNA was extracted from one part and used to detect human HPRT expression as a measurement for human cell metastasis. The remaining lung sections were fixed and embedded in paraffin for hematoxylin and eosin staining.

For the antitumor assay, S18 cells (1 × 10^6 in 100 μl DMEM) were injected near the scapula of 4- to 6-week-old nude mice. The treatment regimen was the same as in the in vivo metastasis assay and lasted approximately 1 month. Tumor length and width were measured with a vernier caliper every other day. Tumor volume was calculated using the formula V = 0.5 × (length × width^2).

Colonies formation assay
Colonies formation assays were performed as previously described (26). S18 and HONE-1 cells were counted and plated in triplicate at 200 cells per well in 6-well plates and cultured for approximately 10 days. The colony formation efficiency was the ratio of the number of colonies formed to the number of cells plated.

Cell apoptosis assay
Cell apoptosis was determined using an Annexin V–FITC apoptosis detection kit (KGA107, Keygen) following the manufacturer’s instructions. Cells were incubated with 20 μmol/L UCB or DMSO for 24 hours. Then, the cells were harvested, washed twice with cold PBS, and resuspended with 500 μl of binding buffer. Cells were stained for 15 minutes at room temperature in the dark with Annexin V–FITC and propidium iodide (PI) and then analyzed by flow cytometry (Beckman Coulter). Cisplatin (DDP), a common clinical drug for NPC, served as a positive control.

Real-time quantitative PCR
Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized using Maxima First Strand cDNA Synthesis Kits (Thermo Scientific) following the manufacturer’s protocol. Quantitative determination of RNA levels was performed in triplicate in three independent experiments. Real-time PCR and data collection were performed with a Bio-Rad CFX96 real-time PCR detection system, and analyses were performed with the Biorad CFX manager 2.1 software. The housekeeping gene β-actin was used as an internal control to normalize expression levels. The primers used for
the amplification of the indicated genes are listed in Supplementary Table S2.

**Western blot analysis**
Western blotting was performed as previously described (27). Briefly, cells were lysed in RIPA lysis buffer on ice, and the clarified lysates were resolved by SDS-PAGE and transferred to polyvinylidenedifluoride membranes for Western blotting using ECL detection reagents (Reoytime). Antibodies against MMP-9, p-p38, p-ERK1/2, and ERK1/2 were obtained from Cell Signaling Technology. The antibody against MMP-2 was from Merck Millipore, and the antibody against α-tubulin was from Santa Cruz Biotechnology.

**Gelatin zymography assay**
MMP-2 and MMP-9 protease activities in the concentrated supernatant medium of S18 or HONE-1 cells were detected by the zymography assay. Briefly, 10% SDS-PAGE gels containing 0.1% gelatin were used to separate the proteins by electrophoresis under nonreducing conditions. Gelatin zymography was performed using an MMP zymography assay kit (Applygen Technologies Inc) according to the manufacturer’s instructions. To activate proteases, the gels were incubated at 37°C for 40 hours in an incubation buffer containing 50 mmol/L Tris–HCl (pH 7.5), 10 mmol/L CaCl2, and 0.02 mmol/L NaN3. The gels were subsequently fixed and stained with 0.25% Coomassie brilliant blue R-250 for 1 hour and washed in 30% methanol and 10% acetic acid to visualize the bands of proteolytic activity.

**Measurement of intracellular ROS levels**
Changes in the intracellular ROS levels were determined by measuring the oxidative conversion of cell-permeable DCFH-DA to fluorescent dichlorofluorescin (DCF) using flow cytometry. Following pretreatment with different concentrations of UCB or DMSO for 24 hours, the cells were collected and adjusted to 1 × 106 cells/mL and incubated with DCFH-DA at 37°C for 20 minutes. Following the incubation, cells were washed 3 times with PBS. The signal intensity of DCF was detected with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

**Statistical analysis**
Receiver operating characteristic (ROC) curve analysis was performed to select the most appropriate cutoff point of UCB levels to stratify patients at a high risk of metastasis. The score closest to the point with both maximum sensitivity and specificity was selected as the cutoff value. The following endpoints were assessed: distant metastasis-free survival (DMFS) and overall survival (OS). The events for DMFS and OS were distant metastasis and death by any cause, respectively. The duration for each endpoint was defined as the time period from the date of diagnosis to the occurrence of the event, or the censored date of follow-up or the date of the final follow-up. These endpoints were analyzed and compared using the Kaplan–Meier method and log-rank tests. Multivariate analyses using the Cox proportional hazards model were performed to test the independence, significance, hazard consistency, and the hazard discrimination. A two-tailed P < 0.05 was considered statistically significant. The analyses were performed using SPSS statistical software (version 16.0, SPSS Inc.).

**Results**
**Univariate analysis of UCB as a prognostic factor for OS and DMFS**
The patients’ characteristics were summarized in Supplementary Table S1. The 5-year OS and DMFS of all patients were 88.2% and 87.7%, respectively. The median of the UCB level was 8.7 μmol/L (interquartile range, 6.9–11.3 μmol/L). The cutoff point of the UCB level for survival outcomes was determined by ROC curve analyses (Supplementary Fig. S1), and a UCB value of 9.7 μmol/L resulted in the most appropriate sensitivity and specificity for DMFS. To identify valuable prognostic factors for NPC, 1,327 histologically diagnosed nonmetastatic NPC patients were recruited for this study. Univariate analysis was used to determine prognostic factors for OS and DMFS in NPC. The analysis revealed that a higher UCB level (≥9.7 μmol/L) was associated with superior OS and DMFS in NPC (P = 0.023 and P < 0.001, respectively; Table 1 and Fig. 1A).

To find whether there is a difference between the UCB levels of males and females, we took a Student’s t test to compare the UCB levels of males and females. The mean of UCB for males was 9.5 μmol/L, while the mean of UCB for females was 8.8 μmol/L. Significant between-group difference was observed (P = 0.004). Serum UCB levels were significantly higher in men than in women. This was consistent with prior studies (15). Then we took separate survival analysis in the two groups. After stratification by gender, UCB levels remained a clinically and statistically significant predictor of DMFS in both groups (Supplementary Table S3). It indicated that biologic effects of UCB were similar between both genders.

**Multivariate analysis indicates that UCB is an independent prognostic factor for DMFS**
We next performed multivariate analysis to investigate whether UCB was an independent predictor for DMFS in NPC. The characteristics, including gender, age, T stage, N stage, overall stage, treatment modality, and serum UCB level were recruited in the multivariate analysis (Table 2). The results showed that UCB ≥ 9.7 μmol/L was a significantly independent predictor for the favorable DMFS [HR, 0.416; 95% confidence interval (95% CI), 0.280–0.618; P < 0.001], while UCB > 9.7 μmol/L was not a significantly independent predictor for the favorable OS (HR, 0.733; 95% CI, 0.520–1.033; P = 0.076). Taken together, our results indicate that UCB is a favorable prognosis marker leading to better DMFS for NPC patients.

**UCB impairs the invasion abilities of NPC cells**
The results obtained from the clinical analysis encouraged us to investigate whether UCB can inhibit the metastasis of NPC. To elucidate the effect of UCB on NPC metastasis in vitro, we tested whether UCB could inhibit the metastasis of the NPC cell lines S18 and HONE-1. Boyden chamber assays showed that 20 μmol/L UCB treatment led to a significant reduction in the number of cells that invaded through the membrane compared with the vehicle control in both S18 and HONE-1 cells (Fig. 1B). However, the migration of NPC cells was not significantly impaired by UCB (Fig. 1B), suggesting that UCB inhibits NPC metastasis by inhibiting NPC cell invasion rather than migration. The inhibition of invasion was also observed in two other NPC cell lines, 5-8F and S26 (Supplementary Fig. S2).
We next evaluated the *in vivo* effects of UCB on NPC cell metastasis. Intraperitoneal injections of 20 mg/kg or 30 mg/kg bilirubin into BALB/c mice each day resulted in an increase of serum UCB levels to 7 to 10 μmol/L, measured at 30 minutes and 6 hours after injection (Supplementary Fig. S3). After 3 days of administration of bilirubin, we injected S18 cells into the lateral tail vein of 8-week-old nude mice and evaluated cancer metastasis to the lungs. Hematoxylin and eosin (H&E) staining showed that intraperitoneal injection of UCB at a dosage of 20 μmol/L UCB does not significantly affect the growth and apoptosis of NPC cells both *in vitro* and *in vivo* (Supplementary Fig. S5A), and 20 μmol/L U0126, a specific MEK inhibitor (Supplementary Fig. S5A), and 20 μmol/L UCB significantly inhibited the expression of MMP-2, and this effect increased when U0126 and UCB treatments were combined. Zymography assays of the cultured media of S18 and HONE-1 cells revealed that both U0126 and UCB treatments decreased MMP-2 expression, and this effect became more distinct when U0126 and UCB treatments were combined (Supplementary Fig. S5B). Moreover, lower levels of ERK1/2 phosphorylation and MMP-2 expression were observed in the NPC lung metastases of nude mice administered UCB (Fig. 3C). Boyden chamber assays showed that the inhibition of ERK1/2 phosphorylation by U0126 decreased the invasion of NPC cells and that coadministration of 20 μmol/L UCB enhanced this effect (Fig. 3D). The decrease in NPC invasion was not due to an inhibition of proliferation because 0.25 μmol/L U0126 did not change the growth rate of S18 or HONE-1 cells (Supplementary Fig. S5C).

### Table 1. Univariate analysis of prognostic factors for patients with NPC

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<th>Factors</th>
<th>5-year (%)</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>5-year (%)</th>
<th>HR (95% CI)</th>
<th>P</th>
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<td>91.6</td>
<td>1.714 (1.144-2.568)</td>
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<td>≤52</td>
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<td>&gt;52</td>
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<td>1.604 (1.66-2.205)</td>
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<td>93.0</td>
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<td>T3/T4</td>
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<td>86.2</td>
<td>1.999 (1.360-2.939)</td>
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<td>N0/N1</td>
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<td></td>
<td>91.5</td>
<td>reference</td>
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<tr>
<td>N2/N3</td>
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<td>1.614 (1.177-2.213)</td>
<td>0.003</td>
<td>85.0</td>
<td>1.827 (1.324-2.521)</td>
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<td>2.475 (1.652-3.709)</td>
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<td>CRT</td>
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<td>&lt;9.7</td>
<td>90.1</td>
<td>0.676 (0.481-0.950)</td>
<td>0.023</td>
<td>93.3</td>
<td>0.387 (0.261-0.574)</td>
<td>&lt;0.001</td>
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**Abbreviations:** RT, radiotherapy; CRT, chemoradiotherapy.
UCB inhibits the invasion of NPC by decreasing the levels of intracellular ROS

Previous studies have suggested that UCB is an antioxidant. Thus, we examined the production of ROS in NPC cells in the absence and presence of UCB treatment. We found that the ROS levels in CNE2-S18 and HONE-1 cells were markedly decreased following 20 μmol/L UCB treatment (Fig. 4A). 

To determine if the inhibition of the invasiveness of NPC cells by UCB was due to a reduction in ROS, we used hydrogen peroxide (H₂O₂) to increase ROS levels in NPC cells treated with UCB and examined whether the invasive potential of the NPC cells recovered. Cell counting assays indicated that low concentrations of H₂O₂ (<50 μmol/L) did not significantly change the growth rate of S18 and HONE-1 cells (Supplementary Fig. S6A). 

ROS analysis showed that 10 μmol/L H₂O₂ treatment could recover the ROS levels in NPC cells treated with UCB (Fig. 4B). 

Figure 1. UCB is a favorable prognostic marker and can inhibit the invasion of NPC cells. A, the OS rate and the DMFS rate were significantly higher in the high-UCB group. B, UCB dramatically reduced the invasive abilities of S18 and HONE-1 cells as determined using Boyden chamber assays. Scale bars, 50 μm. Bars, SD (n = 3). *, P < 0.05; ***, P < 0.001. C, representative H&E staining and wet weight of lungs from mice 7 weeks after tail-vein injection of S18 cells. Scale bars, 500 μm (top) and 100 μm (bottom). **, P < 0.05. D, expression of human HPRT mRNA relative to 18S rRNA in the lungs of tumor-bearing mice. Data are normalized to DMSO-treated mice. Bars, SD (n = 6). *, P < 0.05.
levels of NPC cells treated with 20 μmol/L UCB (Fig. 4B and Supplementary Fig. S6B). Exposure of NPC cells to 20 μmol/L UCB plus 10 μmol/L H2O2 led to a remarkable increase in cell invasion compared with 20 μmol/L UCB alone (Fig. 4C). Western blot and zymography analysis also showed that treating the NPC cells with 10 μmol/L H2O2 restored the expression and activity of MMP-2 and ERK1/2 phosphorylation (Fig. 4D and Supplementary Fig. S6C). Taken together, these results indicate that UCB inhibits the invasion of NPC cells by decreasing intracellular ROS levels and repressing ERK1/2 activation and MMP-2 expression (Supplementary Fig. S6D).

**Discussion**

This study demonstrates for the first time that bilirubin is a favorable and independent prognostic factor for DMFS in NPC patients. Further study revealed that bilirubin can inhibit the metastasis of NPC cells by repressing the phosphorylation of ERK1/2 and subsequent MMP-2 expression via its antioxidation activity.

Until the discovery of its antioxidation capability, bilirubin had long been regarded as just a byproduct of heme metabolism. Extremely high levels of serum bilirubin lead to its accumulation in the brain, causing kernicterus in newborns (33–35). However, moderately high levels of bilirubin (17.1–34.2 μmol/L) are probably beneficial for many diseases, including cancer (13, 16). A case–control study that evaluated the relationship between antioxidant levels and breast cancer risk identified that elevated serum bilirubin levels were correlated with reductions in breast cancer risk (36). In addition, a large cohort study based on a Belgian population noted that higher serum bilirubin levels were associated with lower cancer mortality and concluded that serum bilirubin levels might estimate cancer risk (37). Furthermore, data from the Third National Health and Nutrition Examination Survey of the United States population revealed that serum bilirubin levels had a negative correlation with a history of colorectal cancer (15). All of these studies indicate that higher levels of bilirubin are associated with lower cancer risk and cancer-related mortality. However, few studies evaluated serum bilirubin

**Figure 2.**

UCB inhibits the expression of MMP-2. A and B, real-time quantitative PCR (A) and Western blot (B) analyses showed that 20 μmol/L UCB markedly decreased the expression of MMP-2 in S18 and HONE-1 cells but did not significantly change the expression of MMP-9. Bars, SD (n = 3). **P < 0.05; ***P < 0.01. C, zymography indicated that 20 μmol/L UCB inhibited the activity of MMP-2 in the culture medium of S18 and HONE-1 cells.
as a prognostic marker in patients. To our knowledge, this is the first study that evaluated the association between bilirubin and metastasis. Because bilirubin is an antioxidant and ROS play an important role in tumor metastasis, we hypothesized that bilirubin inhibited the metastasis of NPC cells through its antioxidation activity. As early as the 1950s, bilirubin was reported to protect against the oxidation of lipids, such as vitamin A (38, 39). Then, Stocker and colleagues confirmed that bilirubin was an important physiological antioxidant under physical concentrations (6, 40). As an antioxidant, bilirubin is itself oxidized to biliverdin and then recycled back to bilirubin by biliverdin reductase, reflecting an amplification cycle to control the primary physiological function of bilirubin (41). Bilirubin can protect against 10,000-fold higher concentrations of H2O2, which may be attributed to the role of biliverdin reductase (41). Consistent with previous reports, our results indicate that bilirubin can decrease the ROS levels in NPC cells. Because ROS have been reported to promote metastasis by activating the MAPK pathway (22, 31), we next explored the effect of UCB on MAPK. We found that UCB decreased MMP-2 expression and ERK1/2 phosphorylation by reducing ROS levels.

Metastases represent the end products of a multistep cell-biological process that includes invasion, intravasation, survival in the circulation, arrest at a distant organ site, extravasation, micrometastasis formation, and metastatic colonization (42, 43). Any of these steps can become the therapeutic target in treating tumor metastasis. As an important metabolite in blood circulation, UCB may affect the metastasis of NPC cells at multiple steps. In fact, ROS and MMPs have been reported to be associated with many stages of tumor metastasis, including invasion, intravasation, and extravasation (20, 23). Which steps are affected when UCB impairs the metastasis of NPC cells remains to be explored.

A small number of studies have suggested that UCB may inhibit proliferation and induce apoptosis in cancer cells (28, 29). However, we did not observe an effect on NPC cells until the concentration of UCB reached 100 μmol/L (Supplementary Fig. S4). We speculate the reason for this result is that the sensitivity to the toxicity of UCB is different among tumors. Even within one tumor type, different cell lines are reported to have disparate sensitivities (29). Further studies need to be performed to determine why different tumors have disparate sensitivities to UCB.

In addition to NPC, we also found that elevated UCB at diagnosis was a significant favorable prognostic biomarker correlated with better DMFS in lung cancer (data not shown). Given...

Figure 3.
UCB downregulates the expression of MMP-2 by inhibiting the phosphorylation of ERK1/2. A, S18 (left) and HONE-1 (right) cells were treated with various concentrations (0, 5, 10, and 20 μmol/L) of UCB for 48 hours, and the phosphorylated levels of ERK1/2, p38, and JNK1/2 were determined by Western blot. B, S18 (left) and HONE-1 (right) cells were treated with UCB, ERK1/2 inhibitor U0126, or a combination of UCB and U0126 as indicated. The phosphorylated levels of ERK1/2 and MMP-2 expression were determined by Western blot. C, immunohistochemical analysis of MMP-2 and p-ERK expression in the lungs of mice 7 weeks after tail-vein injection of S18 cells. Scale bars, 100 μm. D, S18 and HONE-1 cells were treated as indicated, and the invasive abilities of the cells were determined using Boyden chamber assays. Scale bars, 50 μm. Bars, SD (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
that ROS and MMPs are important factors in the metastasis of many cancers and that UCB is a vital antioxidant in blood circulation, these results suggest that UCB may play an antime-
tastasis role in many types of tumors.

In conclusion, our results suggest that UCB is a favorable prognostic biomarker correlated with better DMFS in NPC patients and that UCB can inhibit the metastasis of NPC cells both in vitro and in vivo via its antioxidation activity. Elucidating the mechanisms by which UCB inhibits tumor metastasis and researching the potential roles of other antioxidants in blood circulation will provide valuable insight toward understanding tumor metastasis and discovering a novel strategy for the chemo-
prevention of NPC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Xu, J.-X. Bei, M.-Y. Chen, Y.-X. Zeng
Development of methodology: C.-C. Deng, M. Xu, J. Li, X.-L. Luo, R. Jiang, M.-Y. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-C. Deng, J. Li, Y.-J. Zhu, L.-Z. Chen, Q.-S. Feng, M.-Y. Chen
Analysis and interpretation of data (e.g., statistical analysis, biositistics, computational analysis): C.-C. Deng, M. Xu, X.-L. Luo, M.-Y. Chen, Y.-X. Zeng
Writing, review, and/or revision of the manuscript: C.-C. Deng, M. Xu, X.-L. Luo, M.-Y. Chen, Y.-X. Zeng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.-J. Lei, Y.-F. Lian, X. Zou, R. You, J.-X. Bei, M.-Y. Chen
Study supervision: M.-Y. Chen, Y.-X. Zeng

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Figure 4.
UCB decreases intracellular reactive oxygen species levels of NPC cells. A, S18 and HONE-1 cells were treated with DMSO or 20 μmol/L UCB. Intracellular ROS were determined by measuring the oxidative conversion of cell-permeable DCFH-DA to fluorescent DCF using either flow cytometry (top) or fluorescence microscopy (bottom). Scale bars, 50 μm. B, S18 and HONE-1 cells were treated with DMSO, 20 μmol/L UCB or a combination of 20 μmol/L UCB and 10 μmol/L H2O2. Levels of intracellular ROS were determined by flow cytometry. C, S18 and HONE-1 cells were treated as indicated, and the invasive abilities of the cells were determined using Boyden chamber assays. Scale bars, 50 μm. Bars, SD (n = 3). *, P < 0.05; **, P < 0.001. D, S18 and HONE-1 cells were treated as indicated, and the phosphorylated levels of ERK1/2 and MMP-2 expression were determined via Western blot.

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References

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