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Research Paper

Indirect bilirubin impairs invasion of osteosarcoma cells *via* inhibiting the PI3K/AKT/MMP-2 signaling pathway by suppressing intracellular ROS

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HIGHLIGHTS

- Osteosarcoma represents the most prevalent primary malignant bone tumor.
- Primary metastatic patients account for approximately 25% of all osteosarcoma patients, yet their 5-year survival rate is less than 30%.
- IBIL can serve as an independent prognostic predictor for osteosarcoma patients.
- IBIL impairs invasion of osteosarcoma cells via repressing the PI3K/AKT/MMP-2 pathway by suppressing intracellular ROS.
- IBIL is a low-cost prognostic biomarker and a favorable drug target for improving osteosarcoma survival.

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ABSTRACT

Background: Osteosarcoma is most prevalently found primary malignant bone tumors, with primary metastatic patients accounting for approximately 25% of all osteosarcoma patients, yet their 5-year OS remains below 30%. Bilirubin plays a key role in oxidative stress-associated events, including malignancies, making the regulation of its serum levels a potential anti-tumor strategy. Herein, we investigated the association of osteosarcoma prognosis with serum levels of TBIL, IBIL and DBIL, and further explored the mechanisms by which bilirubin affects tumor invasion and migration.

Methods: ROC curve was plotted to assess survival conditions based on the determined optimal cut-off values and the AUC. Then, Kaplan-Meier curves, along with Cox proportional hazards model, was applied for survival analysis. Inhibitory function of IBIL on the malignant properties of osteosarcoma cells was examined using the qRT-PCR, transwell assays, western blotting, and flow cytometry.

Results: We found that, versus osteosarcoma patients with pre-operative higher IBIL (>8.9 μmol/L), those with low IBIL (≤8.9 μmol/L) had shorter OS and PFS. As indicated by the Cox proportional hazards model, pre-operative IBIL functioned as an independent prognostic factor for OS and PFS in total and gender-stratified osteosarcoma patients ($P < 0.05$ for all). In vitro experiments further confirmed that IBIL inhibits PI3K/AKT phosphorylation and downregulates MMP-2 expression *via* reducing intracellular ROS, thereby decreasing the invasion of osteosarcoma cells.

Abbreviations: IBIL, indirect bilirubin; TBIL, total bilirubin; DBIL, direct bilirubin; OS, overall survival; PFS, progression-free survival; ROS, reactive oxygen species; MDA, malondialdehyde; CCK-8, cell counting kit-8; qRT-PCR, real-time quantitative PCR; PVDF, polyvinylidene fluoride; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; SOD, superoxide dismutase; ROC, receiver operative characteristic; AUC, area under curve; SD, standard deviation; HR, hazard ratio; CI, confidence interval; ECM, extracellular matrix; DMSO, dimethyl sulfoxide; H_2O_2, hydrogen peroxide; HIF-1α, hypoxia inducible factor-1α; VEGF, vascular endothelial growth factor; BRNP, PEGylated bilirubin nanoparticles.

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Conclusions: IBIL may serve as an independent prognostic predictor for osteosarcoma patients. IBIL impairs invasion of osteosarcoma cells through repressing the PI3K/AKT/MMP-2 pathway by suppressing intracellular ROS, thus inhibiting its metastatic potential.

1. Introduction

Osteosarcoma, as primary malignant bone tumors, most prevalently occur in children and adolescents, and its most common sites are the proximal humerus (10%), proximal tibia (19%) and distal femur (42%) [1,2]. The annual incidence of osteosarcoma in all age groups is 3.1 per million, with two worrying peaks of incidence occurring in adolescents and the elderly, respectively [1]. The treatment of osteosarcoma has made great progress in the past 40 years, with an increasing 5-year survival rate from 15% to 60% in patients without metastasis due to the application of chemotherapy agents [3,4]. In contrast, traditional chemotherapeutic agents are often less effective in treating metastatic osteosarcoma because of their higher malignancy and greater susceptibility to invasion and regional lymph node or distant metastases [5,6]. It is worth noting that primary metastatic patients account for approximately 25% of all osteosarcoma patients, and unfortunately their 5-year overall survival (OS) remains below 30%, which is a key factor in osteosarcoma mortality and treatment failure [1,5]. Moreover, Enneking surgical criteria, histological subtype along with other prognostic indicators frequently lead to different clinical results at the same tumor stage, reflecting the lack of accuracy [7,8]. Accordingly, unfavorable survival outcomes highlight the need to uncover novel pre-treatment prognostic markers and targeted drugs to evaluate patient's prognosis and improve the therapeutic outcomes achievable with current chemotherapy regimens.

Bilirubin, known as the end product of heme catabolism, was once considered a useless waste that is harmful to humans even in high concentrations [9]. Recently, growing studies reported that bilirubin plays key roles in oxidative stress-related diseases, such as stroke [10], cardiovascular diseases [11], metabolic syndrome [12], respiratory diseases [13] and cancer [14], making the regulation of bilirubin serum levels a promising approach for the therapy of these diseases. Substantial evidence has established oxidative stress and chronic inflammation as vital factors in carcinogenesis and cancer progression [15–17]. Persistent oxidative stress induces chronic inflammation by activating inflammation-related transcription factors, including p53, NF-κB, and Nrf2 [15]. Subsequent chronic inflammation induces the transformation of normal cells into tumor cells by activating inflammatory signaling pathways [16]. Previously, a Belgian population-based cohort study showed that high serum bilirubin levels was linked with improved survival in patients with malignant diseases [18]. Of note, indirect bilirubin (IBIL), accounting for exceeding 80% of total bilirubin (TBIL), exerts potential anti-oxidative, anti-inflammatory and anti-tumor properties through the biliverdin reductase cycle in recent years [19–21]. An inverse correlation occurs between IBIL and the risk of cancer death, such as nasopharyngeal [22], breast [23], lung [24], and colorectal cancers [25]. Besides, *in vitro* experiments have also confirmed that IBIL induces apoptosis in colorectal cancer cells, exerts anti-proliferative effects on human adenocarcinoma cells, and represses the growth of several tumor cells [26–28]. Interestingly, recent reports suggested the involvement of high serum TBIL and direct bilirubin (DBIL) concentrations in an amplified risk contributing to lymph node metastasis and poor prognosis in colorectal tumors [29]. Thus, prognostic significance of pre-operative serum level of bilirubin in patients with osteosarcoma remains elusive. Limited evidence exists as to which of TBIL, IBIL or DBIL can function as a valid prognostic predictor for osteosarcoma patients. The specific anti-tumor mechanisms of serum bilirubin in malignant tumors, especially osteosarcoma, are also still unclear.

This work sought to investigate the relationship between serum level of TBIL, IBIL and DBIL and the prognosis of osteosarcoma based on a retrospective study and further explored the mechanisms by which bilirubin affects tumor invasion and migration in *in vitro* experiments.

2. Methods

This retrospective study was carried out with the ratification of the Ethics Committees of Third Affiliated Hospital of Nanchang University, and First Affiliated Hospital of Nanchang University.

2.1. Study population

The study population comprised 141 patients with diagnosed osteosarcoma treated at First Affiliated Hospital of Nanchang University, and Third Affiliated Hospital of Nanchang University between May 2013 and October 2019. The inclusion criteria of the present study were as follows: with pathological diagnosis of osteosarcoma; without any anti-cancer treatment before serum collection; and with complete clinical data and pathology results. For patients to be excluded, they had known hepatobiliary or pancreatic diseases; severe cardiovascular, renal, hematologic, or autoimmune diseases; clinically confirmed severe inflammatory diseases or infection in the past month; blood transfusion within 4 months; nonsteroidal anti-inflammatory drugs treatment within three months; boosted parameters in hepatic function tests (aspartate aminotransferase $>$ 40 U/L and alanine aminotransferase $>$ 50 U/L); TBIL $>$ 40 μmol /L and $>$ 30 μmol/L in male and female or TBIL $<$ 3 μmol/L for either gender, respectively. The treatment protocol of patients was formulated and performed based on the guideline of the National Comprehensive Cancer Network.

2.2. Data collection and follow-up

Blood samples of patients were drawn prior to breakfast within 2 weeks before surgery. Clinical data from electronic medical records were harvested for the subjects. Upon discharge from treatment, all patients were followed up: once every 3 months during the first two years; once every six months for the next 3–5 years; then every year thereafter until the death finally occurs; or until 1st January 2022. Every follow-up included a radiograph of the surgical site, a physical examination, laboratory tests, as well as a chest computed tomography. OS and progression-free survival (PFS), both obtained mainly *via* telephone surveys or hospital records, were calculated.

2.3. Cell culture

Human osteosarcoma cell lines (MG63, 143B) were gifts from professor Kang (Sun Yat-sen University Cancer Center, China). The 10% FBS-containing DMEM (Biological Industries) was used for cell culture. All cells were maintained in 5% CO_2 at 37 °C and lysed every two days.

2.4. Reagents

The IBIL (MCE, MedChem Express, HY-N0323) and PI3K inhibitor LY294002 (MCE, MedChem Express, HY-10108) were purchased from MCE. Each addition of IBIL was freshly prepared, and IBIL-related experiments were performed under light-protected conditions. Reactive oxygen species (ROS) fluorometric assay kit and malondialdehyde (MDA) colorimetric assay kit were provided by Elabscience (Elabscience Biotechnology Co. Ltd, China).

2.5. Cell counting kit-8 (CCK-8) assay

Following treatment of bilirubin at different concentrations, cells were plated in 24-well plates for incubation. For each measurement, the medium was renewed by 300 μL of new medium, and the plates were subjected to 2 h-incubation. Then, CCK-8 liquid (30 μL) was added to each well for 1 h-incubation after being removed from the 12-well plates under light-proof conditions. The medium was aspirated from the 12-well plate to the 96-well plate, with absorbance examined at 450 nm by using Thermo Scientific Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, China).

2.6. Transwell experiments

Transwell inserts were applied for detecting cell migratory and invasive potential. Cell suspension (200 μL, 1 * 10^5 cells) in serum-free DMEM was supplemented to the upper insert and DMEM (500 μL) containing 10% FBS (Biological Industries, USA) to the lower insert to induce cell migration. Alternatively, 50 μL of mixed matrigel (BD Biosciences, USA) needs to be added to the upper insert 2 h prior to the addition of cells in the invasion assay, then, it was performed using the same procedures as the migration assay. Cells were cultured at 37 °C for 22 h, followed by fixation in paraformaldehyde and staining with 0.4% crystal violet. Images were photographed under a microscope (Olympus, Japan). A triplicate of each experiment was carried out.

2.7. Cell apoptosis assay

AnnexinV-FITC/PI apoptosis detection kit (KGA108, Keygen, China) was applied for cell apoptosis assay. The treated cells by different drugs for 48 h were trypsinized and resuspended in 1×binding buffer. Afterward, cells were immersed in AnnexinV-FITC/PI solution and allowed to incubate without light exposure for half an hour. Samples were analyzed by flow cytometry (Becton-Dickinson, USA) to assess apoptotic cells.

2.8. Real-time quantitative PCR (qRT-PCR)

After 48 h of IBIL treatment, TRIzol™ reagent (Invitrogen, USA) was utilized for total RNA extraction. cDNAs were reversely transcribed using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). The qRT-PCR was carried out using SYBR Premix Ex Taq (Takara, Japan) as descbired in the manufacturer's protocols. The $2-\Delta\Delta Ct$ method, normalized to GAPDH, was utilized to determine the relative expression of genes to be tested. Primer sequences are listed as follows:

GADPH: forward: 5′-GCCACCGTCAAGGCTGAGA-3′,
reverse: 5′-TGGTGAAGGGAACGCCAGT-3′;
MMP-2: forward: 5′-ATTTGGCGGACTGTGACGC-3′,
reverse: 5′-CAGGGTGCTGGCTGAGTAGAT-3′;
MMP-13: forward: 5′-TCCTGATGTGGGTGAATACAATG-3′,
reverse: 5′-GCCATCGTGAAGTCTGGTAAAAT-3′;
TIMP-2: forward: 5′- CCAAAGCGGTCAGTGAGA-3′,
reverse: 5′-TGGTGCCCGTTGATGTTC-3′.

2.9. Western blot assay

Cells were lysed by RIPA (with protease inhibitors) on ice for 30 min. We used the BCA protein quantification kit (Biomiga, PW0104, China) to examine the protein concentration. Protein from each sample was separated and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The 5% skim milk-blocked membrane was probed with primary and secondary antibodies, and then detected by an ECL kit (Tiangen, China). Antibodies against MEK1/2 and p-MEK1/2 were obtained from Cell Signaling Technology, and those against GAPDH, matrix metalloproteinase-2 (MMP-2), MMP-13, tissue inhibitors of MMP-2 (TIMP-2), p-PI3K, PI3K, AKT, p-AKT were obtained from ABMART.

2.10. Measurement of intracellular reactive oxygen species (ROS) levels

Flow cytometry (Becton-Dickinson, USA) was used for assaying ROS levels. After treatment with the dimethyl sulfoxide (DMSO) or IBIL at different concentrations for 48 h, the harvested cells were rinsed twice in PBS. Subsequently, the cells were subjected to staining with ROS probe DCFH-DA for 30 min at ambient temperature avoiding direct light.

2.11. Analysis of oxidation status

Following treatment by DMSO or IBIL at different concentrations for 48 h, cells were subjected to lysis with ultrasound. The superoxide dismutase (SOD) activity and MDA content in osteosarcoma cells were measured as per instructions of the kits to assess oxidative stress and antioxidant status.

2.12. Statistical analysis

SPSS 23.0 software, along with GraphPad Prism version 7.0, was applied for statistical calculations, with $P < 0.05$ as the significance level (2-sided). Categorical variables, presented as percentage, were assessed by chi-squared test. ROC curve was calculated for determining optimal cut-off values and AUC for survival. Kaplan-Meier curves, along with Cox proportional hazards model, were applied for analyzing survival conditions and confirming independent prognostic factors. Experimental data were summarized as mean ± standard deviation (SD) and assessed by *t*-tests.

3. Results

3.1. Clinical characteristics and optimal cut-off values

As indicated in Table 1, the clinical characteristics of included subjects were presented. The osteosarcoma patients (n = 141) enrolled herein included 91 (64.5%) men and 50 (35.5%) women. These patients received surgery and then adjuvant chemotherapy.

Based on ROC curves, we characterized the optimal cut-off values of TBIL (11.5 μmol/L), DBIL (3.2 μmol/L), and IBIL (8.9 μmol/L). Furthermore, the included subjects were partitioned into high or low TBIL (>11.5 μmol/L or ≤11.5 μmol/L, respectively), DBIL (>3.2 μmol/L or ≤3.2 μmol/L, respectively), and IBIL (>8.9 μmol/L or ≤8.9 μmol/L, respectively) groups.

The data presented in Table 1 displayed that TBIL was closely linked with age ($P = 0.001$), and pathological fracture ($P = 0.032$). Significant between-group difference occurred between high or low-DBIL groups with respect to gender ($P < 0.003$) and tumor site ($P = 0.048$). Besides, pre-operative IBIL was linked with tumor site ($P = 0.041$), age ($P < 0.001$), and pathological fracture ($P = 0.011$). No other evident difference was noted between clinical characteristics and high or low TBIL, DBIL, and IBIL groups ($P > 0.05$ for all).

3.2. Prognostic significance of pre-operative TBIL, DBIL, and IBIL

Kaplan-Meier curve analysis results showed that patients in the low IBIL group had shorter OS ($P < 0.001$; Fig. 1M) as well as poorer PFS in relation to those in the higher IBIL group ($P = 0.001$; Fig. 1N). Moreover, the low TBIL group had shorter OS ($P = 0.029$; Fig. 1A) compared with the high TBIL group.

Analyses of the OS and PFS were further conducted (Table 2). Univariate Cox proportional hazards model suggested associations of Enneking stage, local recurrence, pathological fracture, metastasis, TBIL, and IBIL with OS ($P < 0.05$ for all). The multivariate model unveiled local recurrence ($P = 0.002$), metastasis ($P = 0.010$), and IBIL ($P = 0.019$) as independent prognostic factors for OS. For PFS, the univariate model suggested associations of tumor size, Enneking stage, local recurrence, pathological fracture, metastasis, TBIL, and IBIL with PFS (*P*

Table 1
The clinical characteristics of all patients with osteosarcoma based on TBIL, DBIL, and IBIL.

Characteristics		TBIL			DBIL			IBIL		
	Total	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value
Age (years)				0.001			0.185			<0.001
≤20	62	39	23		13	49		42	20	
>20	79	28	51		10	69		25	54	
Gender				0.535			0.003			0.331
Male	91	45	46		21	70		46	45	
Female	50	22	28		2	48		21	29	
Tumor size (cm)				0.094			0.048			0.041
≤7	98	42	56		12	86		41	57	
>7	43	25	18		11	32		26	17	
Tumor site				0.132			0.102			0.132
Extremities	105	46	59		14	91		46	59	
Non-extremities	36	21	15		9	27		21	15	
Histological type				0.184			0.114			0.527
Well-differentiated	88	38	50		11	77		40	48	
Poorly differentiated	53	29	24		12	41		27	26	
Enneking stage				0.453			0.441			0.706
I/II	101	50	51		18	83		49	52	
III	40	17	23		5	35		18	22	
Pathological fracture				0.032			0.105			0.011
Yes	31	20	11		8	23		21	10	
No	110	47	63		15	95		46	64	
Neoadjuvant chemotherapy				0.134			0.807			0.250
Yes	89	38	51		14	75		39	50	
No	52	29	23		9	43		28	24	
Local recurrence				0.580			0.854			0.352
Yes	39	20	19		6	33		21	18	
No	102	47	55		17	85		46	56	
Metastasis				0.519			0.157			0.093
Yes	55	28	27		12	43		31	24	
No	86	39	47		11	75		36	50	

Data were present with Chi-square test. $P < 0.05$ was considered significant.

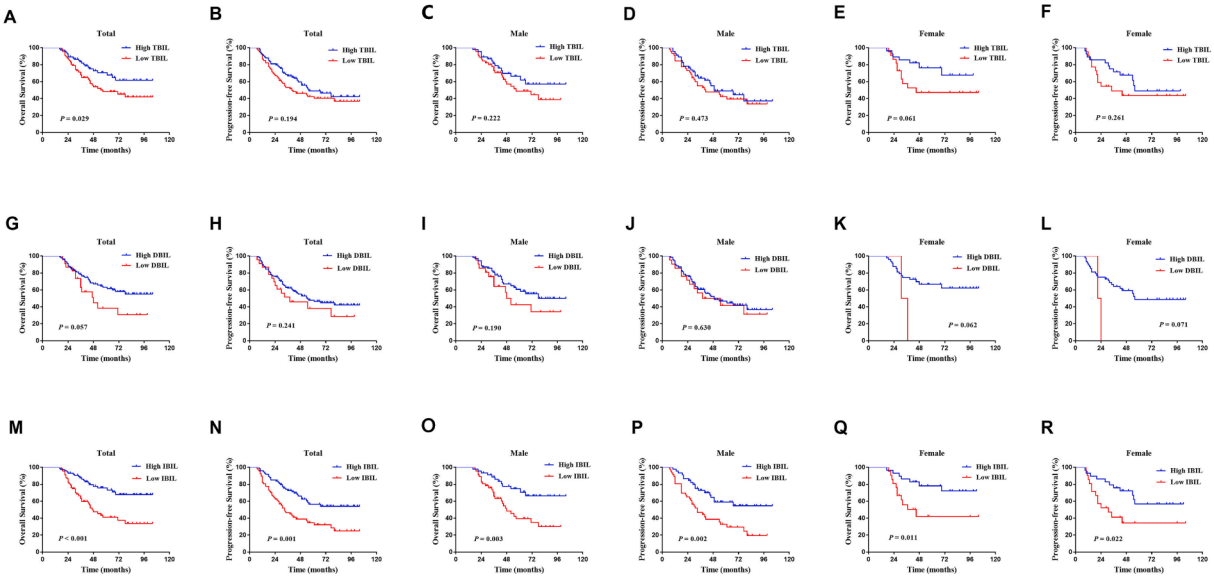

Fig. 1. Kaplan-Meier curves of OS and PFS according to TBIL, DBIL, and IBIL for all patients as well as for gender-stratified patients. (A-F). Kaplan-Meier curves of OS and PFS for total, male, and female patients with osteosarcoma according to preoperative TBIL levels. (G-L). Kaplan-Meier curves of OS and PFS for total, male, and female patients with osteosarcoma according to preoperative DBIL levels. (M–R). Kaplan-Meier curves of OS and PFS for total, male, and female patients with osteosarcoma according to preoperative IBIL levels.

Table 2
Univariate and multivariate analyses for overall and progression-free survival in all patients with osteosarcoma.

Characteristics	OS						PFS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*
Age (years)			0.758						0.507			
≤20	1.000	Reference					1.000	Reference				
>20	1.087	0.640–1.846					1.169	0.737–1.856				
Gender			0.595						0.503			
Male	1.000	Reference					1.000	Reference				
Female	0.859	0.490–1.505					0.846	0.520–1.378				
Tumor size (cm)			0.070						0.033			0.835
≤7	1.000	Reference					1.000	Reference		1.000	Reference	
>7	1.637	0.960–2.790					1.666	1.043–2.660		1.053	0.647–1.713	
Tumor site			0.900						0.739			
Extremities	1.000	Reference					1.000	Reference				
Non-extremities	0.962	0.525–1.763					0.914	0.537–1.555				
Histological type			0.130						0.098			
Well-differentiated	1.000	Reference					1.000	Reference				
Poorly differentiated	1.503	0.887–2.549					1.476	0.930–2.341				
Enneking stage			0.017			0.437			<0.001			0.216
I/II	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
III	1.934	1.124–3.328		1.336	0.643–2.776		2.343	1.470–3.735		1.521	0.783–2.956	
Pathological fracture			0.001			0.329			0.002			0.979
Yes	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
No	0.387	0.225–0.666		0.733	0.393–1.368		0.463	0.283–0.760		0.993	0.562–1.753	
Neoadjuvant chemotherapy			0.078						0.380			
Yes	1.000	Reference					1.000	Reference				
No	1.603	0.949–2.709					1.231	0.774–1.959				
Local recurrence			<0.001			0.002			<0.001			<0.001
Yes	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
No	0.350	0.207–0.595		0.379	0.206–0.697		0.263	0.165–0.419		0.269	0.155–0.466	
Metastasis			<0.001			0.010			<0.001			<0.001
Yes	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
No	0.373	0.219–0.634		0.414	0.213–0.806		0.317	0.199–0.505		0.314	0.173–0.567	
TBIL			0.032			0.459			0.198			
Low	1.000	Reference		1.000	Reference		1.000	Reference				
High	0.557	0.326–0.952		0.735	0.326–1.659		0.741	0.469–1.170				
DBIL			0.062						0.247			
Low	1.000	Reference					1.000	Reference				
High	0.553	0.297–1.030					0.709	0.395–1.269				
IBIL			<0.001			0.019			0.001			0.015
Low	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
High	0.358	0.205–0.623		0.367	0.159–0.848		0.451	0.283–0.720		0.524	0.311–0.883	

Data were analyzed by Cox proportional hazards model. $P < 0.05$ was considered significant.

< 0.05 for all). Additionally, the multivariate model uncovered local recurrence ($P < 0.001$), metastasis ($P < 0.001$), and IBIL ($P = 0.015$) as independent prognostic factors for PFS.

3.3. Association of clinical characteristics with TBIL, DBIL, and IBIL in gender-stratified osteosarcoma patients

We further investigated the relationship between clinical characteristics and TBIL, DBIL and IBIL in gender-stratified patients with osteosarcoma, and showed the results in Table 3. In male patients, TBIL was closely associated with pathological fracture ($P = 0.026$), age ($P < 0.001$), and neoadjuvant chemotherapy ($P = 0.045$). Significant between-group difference occurred between high or low-IBIL groups with respect to tumor size ($P = 0.028$), age ($P < 0.001$), and pathological fracture ($P = 0.010$). In female patients, pre-operative DBIL levels were associated with tumor site ($P = 0.027$). No other evident difference was noted between clinical characteristics and high or low TBIL, DBIL, and IBIL groups ($P > 0.05$ for all).

3.4. Prognostic significance of pre-operative TBIL, DBIL, and IBIL in gender-stratified osteosarcoma patients

Survival analyses of OS and PFS were performed in gender-stratified patients with osteosarcoma and were summarized in Table 4 and Fig. 1.

In male patients, Kaplan-Meier curve analysis results showed that the low IBIL group had shorter OS ($P = 0.003$; Fig. 1O) and PFS ($P = 0.002$; Fig. 1P) compared with the high IBIL group. Subsequently, the univariate analysis further revealed associations of local recurrence, pathological fracture, metastasis, and IBIL with OS ($P < 0.05$ for all). The multivariate model unveiled local recurrence ($P = 0.013$), metastasis ($P = 0.011$), IBIL ($P = 0.029$) as independent prognosis factors for OS. For PFS, the univariate model suggested associations of local recurrence, pathological fracture, metastasis, IBIL, and Enneking stage with PFS ($P < 0.05$ for all). The multivariate model uncovered local recurrence ($P = 0.001$), IBIL ($P = 0.041$), and metastasis ($P < 0.001$), and as independent prognosis factors for PFS.

In female patients, Kaplan-Meier curve analysis exhibited that patients in the low IBIL group had shorter OS ($P = 0.011$; Fig. 1Q) as well as worse PFS ($P = 0.022$; Fig. 1R) in relation to those in the higher IBIL group. Besides, the univariate model suggested associations of local recurrence, IBIL, and metastasis with OS ($P < 0.05$ for all). The multivariate model unveiled local recurrence ($P = 0.033$), and IBIL ($P = 0.023$) as independent prognosis factors for OS. For PFS, the univariate model suggested associations of IBIL, tumor size, local recurrence, Enneking stage, and metastasis with PFS ($P < 0.05$ for all). The multivariate model uncovered local recurrence ($P = 0.002$), IBIL ($P = 0.023$), and metastasis ($P = 0.033$) as independent prognosis factors for PFS.

Table 3
Association of clinical characteristics with TBIL, DBIL, and IBIL in gender-stratified osteosarcoma patients.

Characteristics	Male									Female								
	TBIL			DBIL			IBIL			TBIL			DBIL			IBIL		
	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value
Age (years)			<0.001			0.301			<0.001			0.707			0.721			0.233
≤20	30	13		12	31		32	11		9	10		1	18		10	9	
>20	15	33		9	39		14	34		13	18		1	30		11	20	
Tumor size (cm)			0.059			0.171			0.028			0.804			0.027			0.662
≤7	27	36		12	51		27	36		15	20		0	35		14	21	
>7	18	10		9	19		19	9		7	8		2	13		7	8	
Tumor site			0.328			0.407			0.402			0.250			0.181			0.200
Extremities	29	34		13	50		30	33		17	25		1	41		16	26	
Non-extremities	16	12		8	20		16	12		5	3		1	7		5	3	
Histological type			0.172			0.103			0.446			0.804			0.345			0.851
Well-differentiated	23	30		9	44		25	28		15	20		2	33		15	20	
Poorly differentiated	22	16		12	26		21	17		7	8		0	15		6	9	
Enneking stage			0.280			0.503			0.224			0.856			0.392			0.314
I/II	34	30		16	48		35	29		16	21		2	35		14	23	
III	11	16		5	22		11	16		6	7		0	13		7	6	
Pathological fracture			0.026			0.333			0.010			0.709			0.181			0.617
Yes	16	7		7	16		17	6		4	4		1	7		4	4	
No	29	39		14	54		29	39		18	24		1	41		17	25	
Neoadjuvant chemotherapy			0.045			0.785			0.159			0.709			0.529			0.851
Yes	22	32		13	41		24	30		16	19		1	34		15	20	
No	23	14		8	29		22	15		6	9		1	14		6	9	
Local recurrence			0.766			0.503			0.872			0.631			0.380			0.189
Yes	14	13		5	22		14	13		6	6		1	11		7	5	
No	31	33		16	48		32	32		16	22		1	37		14	24	
Metastasis			0.760			0.442			0.338			0.594			0.479			0.176
Yes	21	20		11	30		23	18		7	7		1	13		8	6	
No	24	26		10	40		23	27		15	21		1	35		13	23	

Data were present with Chi-square test. $P < 0.05$ was considered significant.

Table 4
Univariate and multivariate analyses for overall and progression-free survival in male and female patients with osteosarcoma.

Characteristics	Male												Female											
	OS						PFS						OS						PFS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*	HR	95 %CI	*P*
Age (years)			0.895						0.438						0.627						0.806			
≤20	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference				
>20	1.044	0.551–1.978					1.248	0.713–2.186					1.276	0.478–3.402					1.109	0.485–2.536				
Tumor size (cm)			0.306						0.323						0.134						0.031			0.810
≤7	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference		1.000	Reference	
>7	1.406	0.733–2.696					1.339	0.751–2.389					2.040	0.802–5.189					2.441	1.084–5.498		1.130	0.417–3.058	
Tumor site			0.485						0.521						0.511						0.998			
Extremities	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference				
Non-extremities	0.778	0.384–1.575					0.819	0.445–1.508					0.658	0.189–2.292					0.999	0.296–3.365				
Histological type			0.356						0.195						0.222						0.462			
Well-differentiated	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference				
Poorly differentiated	1.351	0.713–2.558					1.446	0.828–2.526					1.809	0.698–4.691					1.378	0.587–3.233				
Enneking stage			0.086						0.029			0.371			0.093						0.002			0.382
I/II	1.000	Reference					1.000	Reference		1.000	Reference		1.000	Reference					1.000	Reference		1.000	Reference	
III	1.786	0.922–3.461					1.895	1.069–3.362		1.460	0.637–3.344		2.262	0.874–5.856					3.561	1.583–8.010		1.955	0.434–8.801	
Pathological fracture			0.005			0.699			0.007			0.899			0.101						0.240			
Yes	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference					1.000	Reference				
No	0.393	0.206–0.751		0.864	0.412–1.812		0.450	0.252–0.805		0.957	0.483–1.893		0.418	0.148–1.187					0.552	0.205–1.488				
Neoadjuvant chemotherapy			0.067						0.154						0.765						0.523			
Yes	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference				
No	1.820	0.959–3.453					1.500	0.859–2.618					1.162	0.435–3.102					1.352	0.536–3.408				
Local recurrence			0.003			0.013			<0.001			0.001			0.006			0.033			<0.001			0.002
Yes	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
No	0.372	0.196–0.707		0.430	0.221–0.836		0.349	0.196–0.620		0.317	0.156–0.642		0.274	0.108–0.696		0.357	0.138–0.920		0.174	0.076–0.399		0.185	0.063–0.541	
Metastasis			0.002			0.011			<0.001			<0.001			0.042			0.106			0.001			0.033
Yes	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
No	0.360	0.186–0.698		0.410	0.203–0.826		0.307	0.175–0.539		0.295	0.149–0.584		0.380	0.150–0.966		0.457	0.177–1.180		0.264	0.118–0.592		0.221	0.055–0.884	
TBIL			0.228						0.477						0.070						0.266			
Low	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference				
High	0.670	0.349–1.285					0.817	0.468–1.426					0.415	0.160–1.076					0.634	0.283–1.417				
DBIL			0.196						0.633						0.084						0.093			
Low	1.000	Reference					1.000	Reference					1.000	Reference					1.000	Reference				
High	0.629	0.311–1.270					0.853	0.445–1.635					0.267	0.060–1.196					0.277	0.062–1.240				
IBIL			0.005			0.029			0.003			0.041			0.016			0.023			0.027			0.023
Low	1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference		1.000	Reference	
High	0.375	0.189–0.744		0.447	0.217–0.920		0.421	0.236–0.752		0.502	0.259–0.973		0.303	0.115–0.799		0.318	0.119–0.852		0.398	0.176–0.902		0.368	0.156–0.870	

Data were analyzed by Cox proportional hazards model. $P < 0.05$ was considered significant.

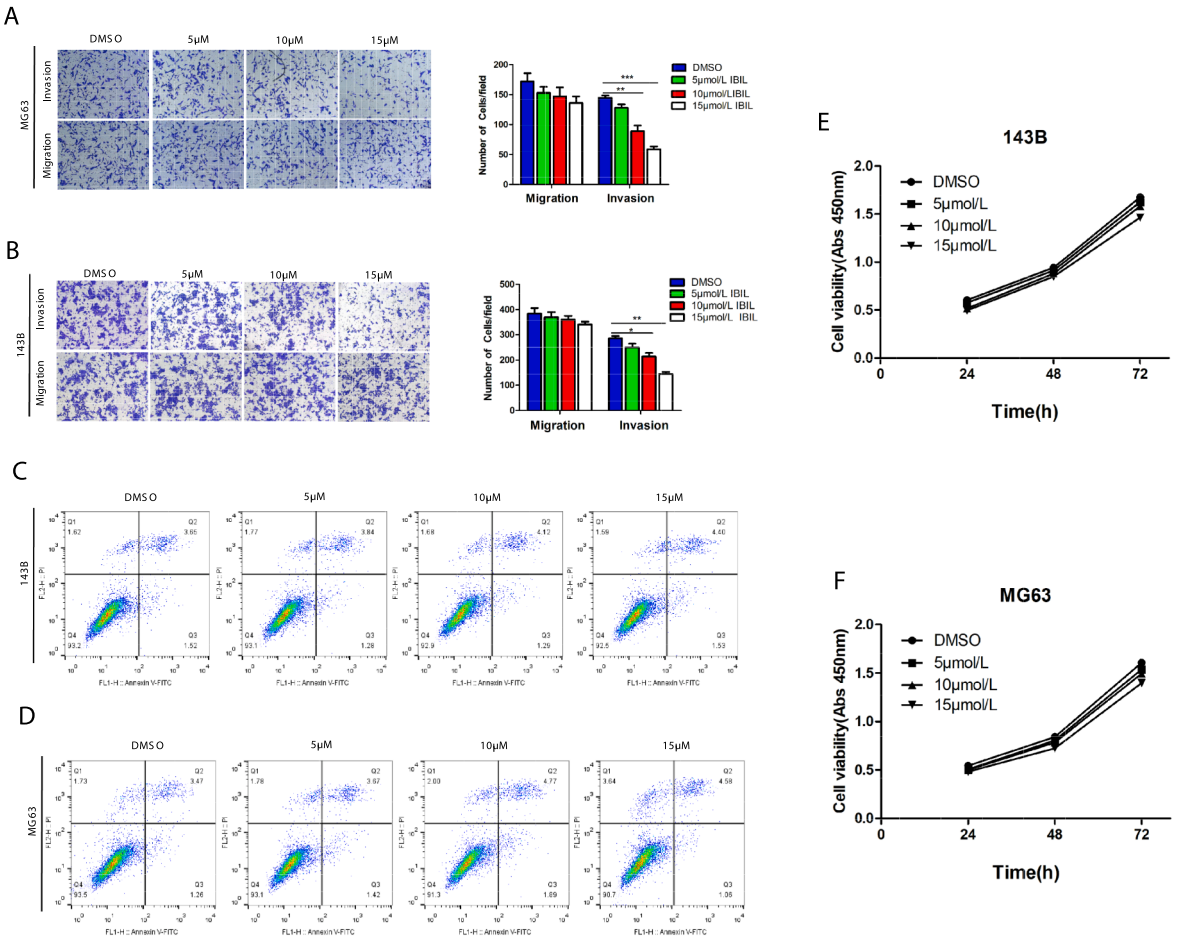

Fig. 2. IBIL downregulates the expression of MMP-2. (A). IBIL dramatically reduced the invasive abilities of 143B cells as determined using Boyden chamber assays. Scale bars represent 50 μm. Bars, SD (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B). IBIL dramatically reduced the invasive abilities of MG63 cells as determined using Boyden chamber assays. Scale bars represent 50 μm. Bars, SD (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (C). Detection of apoptosis in 143B treated with DMSO or IBIL for 48 h. Cell apoptosis results were tested by flow cytometry. (D). Detection of apoptosis in MG63 treated with DMSO or IBIL for 48 h. Cell apoptosis results were tested by flow cytometry. (E). MG63 cells were treated with different concentrations of IBIL and the cell viability was measured at 24 h, 48 h and 72 h, by CCK-8 assays, compared with controls, IBIL essentially did not affect osteosarcoma cell growth. (F).143B cells were treated with different concentrations of IBIL and the cell viability was measured at 24 h, 48 h and 72 h, by CCK-8 assays, compared with controls, IBIL essentially did not affect osteosarcoma cell growth.

3.5. IBIL impairs the invasion abilities of osteosarcoma cells

Since our clinic analysis above has identified IBIL as a prognosis marker of osteosarcoma, we sought to explore whether IBIL affected cancer cell migration and invasion. Based on Transwell assay results, IBIL reduced the invasion of MG63 cells and 143B cells. The inhibition effect was more pronounced with the increase of IBIL concentration. The 15 μmol/L IBIL treatment significantly reduced the number of the filtered cells (Fig. 2A&2B). However, IBIL failed to notably impair the migratory capacity of osteosarcoma cells (Fig. 2A&2B). It indicated that the IBIL inhibits cell metastasis by suppressing cell invasion ability.

IBIL may inhibit tumor cell growth and induce cell apoptosis, as revealed by a prior study [27]. Here, however, our results showed that IBIL even up to 15 μmol/L has no or little effect on the proliferative and apoptotic capacities of osteosarcoma cells (Fig. 2C-2F). Altogether, these results indicated that IBIL played an anti-metastasis effect on the osteosarcoma cells by inhibiting their invasive ability.

3.6. IBIL downregulates MMP-2 expression

MMPs, such as MMP-2 and MMP-13, have been documented to be crucial for tumor metastasis. It can mediate cellular degradation of host extracellular matrix (ECM), control tumor neovascularization, regulate cell adhesion and motility, and modulate tumor cell growth by affecting intracellular signaling, and are directly or indirectly involved in physiological and pathological processes. Meanwhile, the activity of MMPs were regulated by TIMPs, and then we evaluated the effect of IBIL on TIMPs expression. The effect of IBIL on the mRNA and protein expression of MMPs and TIMPs were investigated with qRT-PCR and western blot assay. As shown in Fig. 3A&3B, treatment of IBIL appreciably reduced the expression of MMP-2, yet significantly upregulated TIMP-2 expression in MG63 and 143B cells in a dose-responsive manner. However, MMP-13 expression was not significantly affected by IBIL. Thus, IBIL may affect osteosarcoma metastasis by suppressing MMP-2 expression.

3.7. IBIL diminishes MMP-2 levels by repressing PI3K/AKT phosphorylation

Prior evidence has shown that the invasive ability of osteosarcoma cells and MMP-2 expression are associated with PI3K-AKT signaling dysregulation [30]. This allowed us to wonder whether IBIL affects PI3K-AKT in osteosarcoma cells. The result was shown in Fig. 3C, compared with DMSO treatment group, treatment of IBIL decreased the phosphorylation of PI3K/AKT significantly but not MAPK phosphorylation.

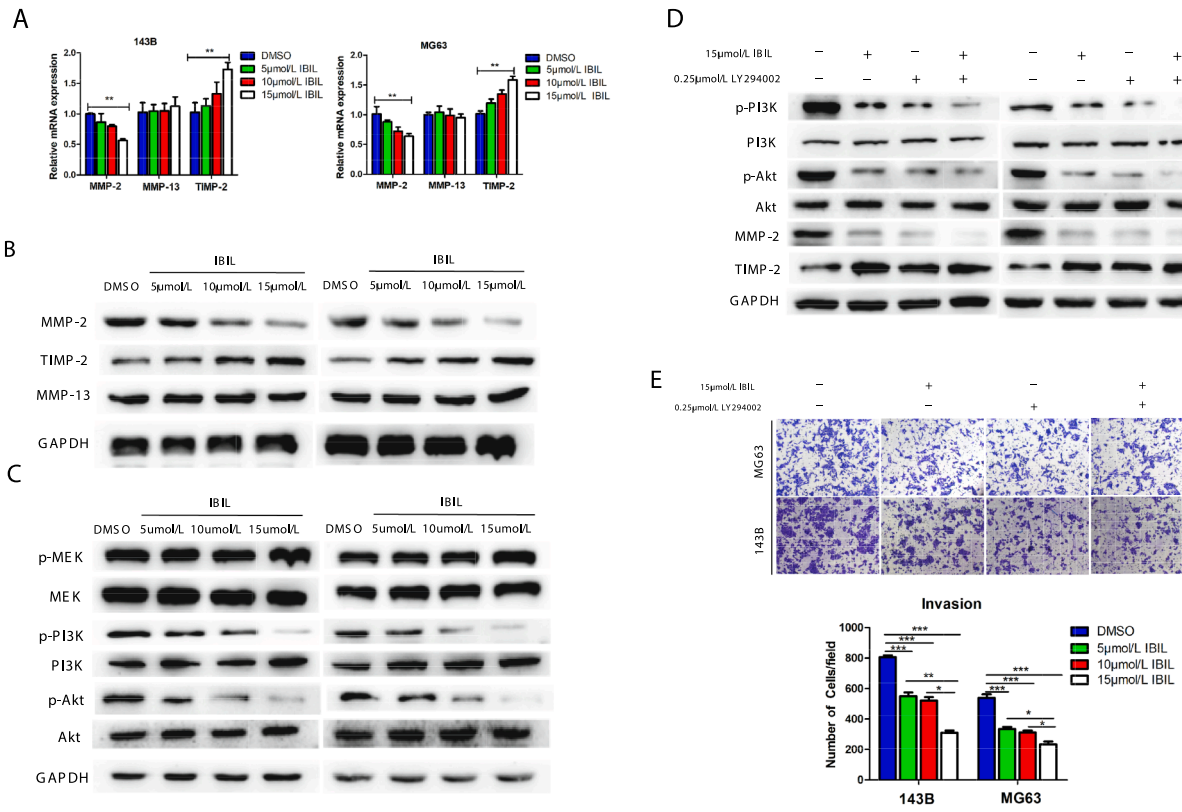

Fig. 3. IBIL downregulates MMP-2 expression by inhibiting the phosphorylation of PI3K/AKT. (A). qRT-PCR showed that IBIL inhibited the expression of MMP-2 and promoted the expression of TIMP-2, but did not significantly change the expression of MMP-13 in 143B and MG63 cells. Bars, SD (n = 3). $^{**}P < 0.01$. (B). Western blot analyses showed that 15 μmol/L IBIL markedly decreased the expression of MMP-2 and upregulated the expression of TIMP-2 in 143B and MG63 cells, but did not significantly change the expression of MMP-13. Bars, SD (n = 3). (C). IBIL downregulates the expression of MMP-2 by inhibiting the phosphorylation of PI3K/AKT. 143B (left) and MG63 (right) cells were treated with various concentrations (0, 5, 10, and 15 μmol/L) of IBIL for 48 h, and the phosphorylated levels of MEK, PI3K, and AKT were determined by western blotting. (D). 143B (left) and MG63 (right) cells were treated with IBIL, PI3K inhibitor LY294002, or a combination of IBIL and LY294002 as indicated. The phosphorylated levels of PI3K/AKT, and the expression of TIMP-2 and MMP-2 were determined by western blotting. (E). 143B and MG63 cells were treated as indicated, and the invasive abilities of the cells were determined using Boyden chamber assays. Scale bars represent 50 μm. Bars, SD (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.001$.

It was previously reported that in many tumors, MMP-2 expression is regulated by PI3K phosphorylation. We next examined whether PI3K phosphorylation can impact MMP-2 expression in osteosarcoma cells. As show in Fig. 3D, both 15 μmol/L IBIL, and LY294002, a specific PI3K inhibitor significantly inhibited MMP-2 expression and upregulated TIMP-2 expression. The inhibited MMP-2 expression and upregulated TIMP-2 expression were most pronounced when IBIL and LY294002 were used together. These results suggested that IBIL may affect MMP-2 expression by inhibiting PI3K/AKT phosphorylation. Transwell assays further confirm our findings. As shown in Fig. 3E, the attenuation of PI3K phosphorylation by LY294002 diminished the invasive ability of osteosarcoma cells, which was enhanced by co-administration of 15 μmol/L IBIL.

3.8. IBIL inhibits the invasion of OS by diminishing intracellular ROS levels

ROS can promote PI3K/AKT phosphorylation and tumor invasion. Bilirubin has antioxidant activity, and thus we examined whether IBIL can decreased ROS production in osteosarcoma cells. We also used the fluorescent DCFH-DA probe to evaluated ROS levels and whether IBIL can decreased ROS production in osteosarcoma cells. We found that with 15 μmol/L IBIL treatment the ROS levels in 143B and MG63 cells were markedly decreased (Fig. 4A&4B). In addition, we further evaluated the relationship between IBIL and oxidative stress. Compared with the DMSO group, 15 μmol/L IBIL significantly stimulated the activity of SOD and inhibited the production of MDA. Hence, IBIL has antioxidant activity which affect oxidative stress in cells (Fig. 4C). To determine if the inhibited osteosarcoma cell invasion by IBIL was resulted from ROS reduction, we applied H_2O_2 to boost ROS production in IBIL-treated osteosarcoma cells and evaluated whether the osteosarcoma cell invasion was recovered. In the Transwell assays (Fig. 4D), compared with IBIL treatment alone, the invasive ability of osteosarcoma cells treatment with IBIL and H_2O_2 was enhanced. Next, we want to know whether H_2O_2 can regulate the levels of MMP-2 and TIMP-2 as well as PI3K phosphorylation. Western blot assay showed that regulation the levels of MMP-2 and TIMP-2, and the PI3K phosphorylation could be restored by H_2O_2 treatment (Fig. 4E). These results suggested that IBIL inhibits osteosarcoma invasion by decreasing intracellular ROS levels.

5. Discussion

Our study identified IBIL as an independent prognostic factor for osteosarcoma, and relatively elevated pre-operative IBIL levels suggest good OS and PFS for osteosarcoma. Furthermore, we concluded that IBIL can impair invasion capability of osteosarcoma cells by repressing PI3K/AKT phosphorylation and MMP-2 expression through reducing intracellular ROS, thereby inhibiting its metastasis.

Previous multiple investigations have shown a positive correlation between prognosis of tumors and bilirubin serum levels, consistent with our finding. Yao et al. [22] concluded that IBIL might independently act as a protective prognostic factor for PFS in patients with high-grade

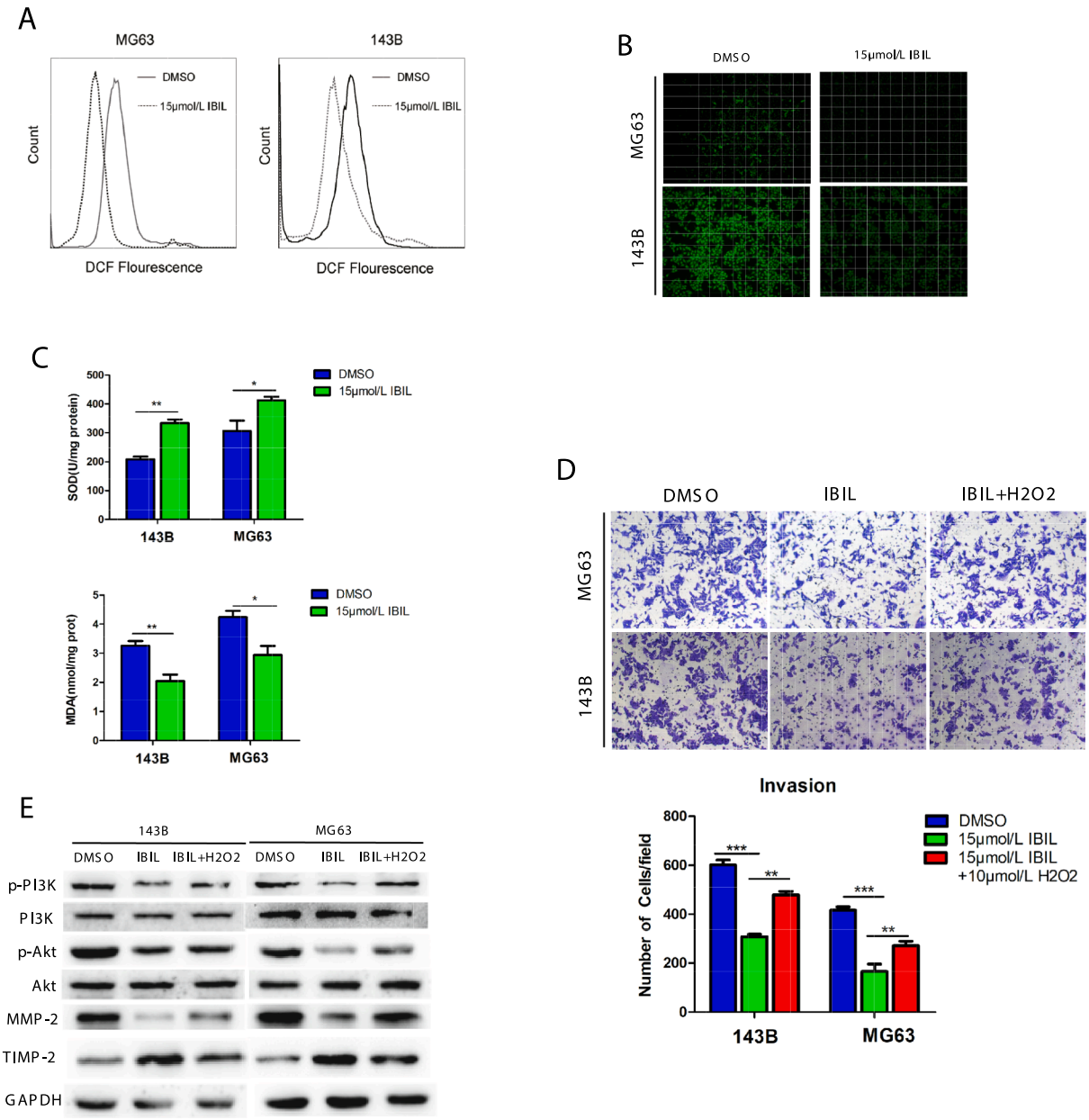

Fig. 4. IBIL inhibits the invasion of osteosarcoma by decreasing the levels of intracellular ROS. (A-B). IBIL decreases intracellular reactive oxygen species levels of osteosarcoma cells. MG63 and 143B cells were treated with DMSO or 15 μmol/L IBIL. Intracellular ROS were determined by using flow cytometry or fluorescence microscopy. (C) 143B and MG63 cells were treated as indicated, and SOD activity and MDA content were calculated for each group of cells using Reagent Kits. $^{*}P < 0.05$; $^{**}P < 0.01$. (D). 143B and MG63 cells were treated as indicated, and the invasive abilities of the cells were determined using Boyden chamber assays. Scale bars represent 50 μm. Bars, SD (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (E). 143B and MG63 cells were treated as indicated, and the phosphorylated levels of PI3K/AKT as well as the expression of TIMP-2 and MMP-2 were determined *via* western blotting.

nasopharyngeal carcinoma. Besides, Li et al. [24] showed that OS of patients suffering from non-small cell lung cancer was significantly prolonged in the high IBIL group. However, there is a lack of basic experiments to reveal the potential pathological mechanisms of preoperative IBIL suggestive of a better tumor prognosis as described above. As one of the few studies that have explored the mechanism, Deng et al. [31] have demonstrated that IBIL improves the prognosis of nasopharyngeal carcinoma by inhibiting MEK/ERK1/2 activation and MMP-2 expression and ROS production. Yet, whether the mechanism revealed by their study is applicable to osteosarcoma, we conducted further experiments to explore.

Notably, our study for the first time demonstrated that IBIL, due to its antioxidant properties, was able to inhibit downstream MMP-2 expression by inhibiting phosphorylation of PI3K/AKT rather than MEK/ERK1/2, thereby inhibiting invasion of osteosarcoma cells. Previously, Stocker et al. [20] and Sedlak et al. [32] reported IBIL as an endogenous antioxidant that may provide cytoprotection. In terms of its mechanism, it may be involved in scavenging ROS, resulting in a decrease in oxidative stress [33]. We hypothesized that IBIL which serve as an antioxidant could exert cytoprotective effects by scavenging oxidative stress, thereby inhibiting osteosarcoma cell invasion. Further experiments have confirmed our hypothesis that IBIL can decrease the ROS levels in osteosarcoma cells, as concurring with previous studies [27,34]. ROS, induced by physiological biochemical reactions, can work as prominent intermediate of cellular physiological and pathological activities to modulate pathways. Zhang et al. [35] reported that ROS can activate such pathways as RAS/RAF/ERK1/2 and PI3K/AKT in human colorectal adenocarcinoma. Another work of He et al. [36] highlighted

that ROS may activate AKT and ERK1/2, and expedite the levels of HIF-1α and VEGF in human bronchial epithelial cells. Besides, Yang et al. [37] found that ROS may interfere with cell cycle progression and chromosome stability to exert carcinogen activity. All the studies mentioned above indicated that the association of ROS and proliferation, invasion and metastasis of tumor cells. We next explored whether IBIL could eliminate ROS to inhibit these signaling pathways, thereby conferring the antioxidant and anti-tumor effects. The results revealed that IBIL decreased MMP-2 expression mainly *via* inactivation of the PI3K/AKT pathway, other than affecting other signaling pathways. Of importance, H_2O_2-induced boosting of ROS production in cells following IBIL treatment restored the levels of MMP-2. Meanwhile, the PI3K inhibitor specifically repressed the PI3K/AKT pathway activation and reduced MMP-2 levels in osteosarcoma cells. Altogether, our data indicated that IBIL *via* inhibits the activation of the PI3K/AKT/MMP-2 pathway *via* reducing ROS levels.

In this study, clinical and basic research have been relatively closely integrated. We analyzed the association of TBIL, DBIL, and IBIL with survival in patients with osteosarcoma respectively, rather than just assessing one of the bilirubin indicators alone. Compared to other studies that only investigated the relationship between IBIL and tumor outcomes at the clinical aspect, we also validated it at the cellular level to further confirm our results. As a prognostic indicator, IBIL is more suitable for the therapeutic strategies of osteosarcoma in clinical scenario with few resources. This study offered the possible optimal cut-off value to aid the clinicians in identifying the high-risk individuals with unsatisfactory prognosis, target patients requiring close post-operative monitoring and adjuvant chemotherapies, which may ultimately increase survival conditions. Further, we finally found that IBIL inhibits osteosarcoma invasion by inhibiting PI3K/AKT/MMP-2, which may provide a new direction for future therapeutic drug targets.

Certainly, our study inevitably has some limitations. Firstly, this research, as a retrospective design, is susceptible to bias in data selection and analysis. Secondly, due to the rare characteristics of osteosarcoma, the sample size included remains limited, and the existing data may not reflect the clinical situation of all osteosarcoma patients. Thirdly, the current study has only performed in vitro experiments, and it still lacks in vivo experiments to explore the role of IBIL in inhibiting the ability of osteosarcoma invasion. Finally, this study is limited by objective conditions failing to explore the effect of the matrix on the distribution and transport of circulating molecules such as IBIL, although a more physiological 3-D system or preclinical work would be more convincing. Therefore, our findings still need the large sample size and multi-center clinical studies, as well as more refined in vivo and in vitro experiments to further verify the conclusions we obtained. Moreover, IBIL, as an endogenous antioxidant, can exert the anti-tumor effects, however, it also has drawbacks, mainly including water insolubility and easily oxidized, limiting the clinical application of IBIL. Promisingly, since nanotechnology is widely applied in pharmaceutics, more and more compounds with inherent defects have achieved nanometerization development, which enables IBIL to overcome the disadvantages in the practical application. In the past decade, growing literatures have reported the use of PEGylated bilirubin nanoparticles (BRNP) in the tumor treatment, suggesting BRNPs a novel therapeutical strategy for osteosarcoma [38–42].

6. Conclusion

In conclusion, this study confirmed that osteosarcoma patients with relatively high serum IBIL levels were associated with better OS and PFS. Further study found that IBIL can impair invasion of osteosarcoma cells by repressing the PI3K/AKT/MMP-2 signaling pathway through reducing ROS levels, and ultimately inhibiting its metastatic potential. Current work may help clinicians recognize IBIL as a low-cost prognostic biomarker and favorable drug target for improved osteosarcoma survival.

Ethics and Consent to participate

Ethics committee belonging to Third Affiliated Hospital of Nanchang University and First Affiliated Hospital of Nanchang University ratified the current study (grant number: KY2022054 & 2021101201), which was implemented with *Declaration of Helsinki*.

Consent for publication

Not applicable.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Author contributions

Xuhui Yuan and Cong Ma and Jiayu Li contributed equally to this work. Xiaobin Lv and Liao Qi designed the current study and amended the paper. Xuhui Yuan and Cong Ma and Jiayu Li were the major writer of the paper, tested the entire index for all samples, and was responsible for the statistical analysis. Junhong Li created all tables and figures, and provided suggestions for important intellectual content. Xuhui Yuan, Cong Ma, Jiayu Li, Junhong Li, Ronghui Yu, Feng Cai, Gaoyang Qu, and Bo Yu performed the experiments and collected the data. Ronghui Yu, Lang Liu, Duo Zeng, and QuanHui Jiao supervised the study and performed the literature search and clinical follow-up. All authors discussed the results, and Xuhui Yuan, Cong Ma, and Junhong Li made critical revisions to the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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