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TopBP1 promotes malignant progression and correlates with poor prognosis in osteosarcoma

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Abstract. – OBJECTIVE: Topoisomerase IIβ binding protein 1 (TopBP1) is involved in DNA damage and replication checkpoint and has been shown to be related to tumorigenesis in many cancer types. This study aimed to evaluate the biological role and clinicopathological significance of TopBP1 in OS.

PATIENTS AND METHODS: TopBP1 expression in sarcoma patients was determined through the Oncomine database, and the prognostic role of TopBP1 expression was assessed in a retrospective cohort study. CCK-8 assay and colony formation assay were employed to evaluate the effect of TopBP1 on proliferation and chemoresistance in OS cells. Cell apoptosis and cell cycle assay were used to assess the effect of TopBP1 on apoptosis and cycle of OS cells.

RESULTS: We observed that TopBP1 expression was elevated not only in OS, but also in other sarcoma types including myxofibrosarcoma, liposarcoma, and leiomyosarcoma. Knockdown of TopBP1 using small interfering (si) RNA blocked cell proliferation and colony formation ability, and caused cell apoptosis as well as G1-phase arrest in OS cells. Moreover, TopBP1 knockdown decreased the chemoresistance of OS cells to both doxorubicin and cisplatin. Lastly, the retrospective cohort study showed that high TopBP1 expression was not only associated with high local recurrence and low necrosis rate, but also correlated with poor overall survival and disease-free survival of OS patients.

CONCLUSIONS: Our findings indicate that TopBP1 contributes to the cell survival and chemoresistance to doxorubicin and cisplatin of OS, suggesting TopBP1 may serve as a novel target for inhibition of progression and chemotherapeutic resistance in OS patients.

Key Words
TopBP1, Osteosarcoma, Chemoresistance, Prognosis.

Introduction

Osteosarcoma (OS), a malignant bone-forming tumor affecting mainly children and adolescents, is the second highest cause of cancer-related death in childhood and adolescents[1]. Although the therapeutic strategy of OS has improved due to the implantation of multi-agent chemotherapy in combination with improved surgical techniques in the 1970s, the outcome of patients has not changed dramatically with the 5-year survival rate of non-metastatic OS stagnating at 60-70% and less than 20% for metastatic disease over the past decades[2]. Metastasis and chemoresistance still remain tough obstacles to successful treatment of OS[3]. So, there is an urgent need to elucidate the mechanisms underlying the malignant development and identify novel biomarkers responsible for mediating tumor progression and chemotherapeutic resistance.

DNA topoisomerase II, a clastogen inducing carcinogenic chromosomal aberrations by relaxing the supercoiled DNA and resealing the DNA backbone, is a target of widely used anticancer drugs[4,5]. As an interacting partner for topoisomerase IIβ, topoisomerase IIβ binding protein 1 (TopBP1) contains repeats of BRCA1 (breast cancer gene 1) carboxyl-terminal (BRCT) motifs that are involved in DNA damage repair and cell cycle checkpoints[6-8]. TopBP1 also colocalizes with 53BP1 (p53 binding protein 1) and NBS1 (Nijmegen breakage syndrome protein 1), and interacts with transcription factor E2F1, human papillomavirus type 16 (HPV16) transcription/replication factor E2, DNA polymerases ε and checkpoint protein hRad9, suggesting TopBP1 not only plays a role in DNA replication and checkpoint activation, but also possesses activity in tran-

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scriptional regulation[7,9-11]. It has been reported that TopBP1 is abnormally overexpressed in many tumors and exerts a variety of functions on biological features of tumor, such as cell proliferation, apoptosis and chemoresistance[12]. Nevertheless, the functional role of TopBP1 in prognosis, tumorigenesis, progression of OS has not yet been fully elucidated.

In this work, we firstly investigated the expression of TopBP1 in sarcoma tissues and cell lines, and then detected whether TopBP1 could influence the cell proliferation, apoptosis and cell cycle distribution of OS cells. Further *in vitro* assay was also employed to identify whether TopBP1 has any impact on the chemosensitivity to doxorubicin and cisplatin in OS cells. Lastly, we explored the clinical role of TopBP1 in a retrospective cohort study enrolling 117 OS patients. Taken together, our study indicates that TopBP1 may be a promising therapeutic target and serve as a biomarker to predict the prognosis of OS.

Patients and Methods

Patients

A total of 117 OS patients who underwent surgery and received standard chemotherapy at the Shanghai Jiao Tong University Affiliated Sixth People's Hospital between September 2007 to November 2013, were enrolled in our study. The chemotherapeutic responses were defined as inferior (<90% tumor necrosis) and favorable (≥90% tumor necrosis) based on criterions suggested by Huvos et al[14]. Written informed consent was given by all participating patients and the Ethics Review Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital approved this study.

Bioinformatics Analysis Through Oncomine Databases

Oncomine database (http://www.oncomine.org) was used to explore the mRNA expression difference of TopBP1 between sarcoma and matched normal tissues as previously described[13]. The filtering conditions were restricted as follows: gene: TopBP1; cancer type: sarcoma; analysis type: cancer *vs.* normal analysis; data type: mRNA; fold change > 2; gene rank: top 10%, p-value < 0.05.

Cell Culture and Reagents

The human OS cell lines (U2OS, MG63, SaoS2, MNNG/HOS and 143B) and a normal osteoblast cell line hFOB1.19 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) as well as streptomycin (100 μg/ml) and penicillin (100 U/ml). All cells were cultured at 37°C in 5% CO_2. Doxorubicin and cisplatin were purchased from Selleck Chemicals (Houston, TX, USA).

Quantitative RT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was converted to complementary DNA with the PrimeScript RT-PCR Kit. PCR was performed using SYBRGreen Kit (TaKaRa, Otsu, Shiga, Japan) and the $2^{-\Delta\Delta ct}$ method was used to calculate the relative mRNA levels. The primers for TopBP1 were as followed: forward 5'-AAGAGTTTCCTTGTTTTGGG-3' and reverse 5'-CATGCCTTTCTTTGCATTGG -3'. Results were normalized to β-actin levels determined with primers as follows: forward 5'-GTGGACATCCGCAAAGAC-3' and reverse 5'-AAAGGGTGTAACGCAAC TA-3'.

Transient siRNA Transfection

The small interfering RNA (siRNA) against TopBP1 and negative control siRNA were synthesized by Biofavor Co., Ltd., (Wuhan, China). The sequences of the synthesized oligonucleotides were as follows: scramble control siRNA: 5'-CUUACGCUGAGUACU UCGATT-3' and TopBP1: ACCGAGUACGCCACUCUCA-3'. 3×10^5 cells were seeded into each chamber of the six-well plate and grew until 70% confluence. Then Lipofectamine™ (Invitrogen, Carlsbad, CA, USA) was used to mediate siRNA into cells according to the instruction. The expression level of TopBP1 was confirmed by qRT-PCR and Western blot.

Western Blotting

Proteins were extracted by radioimmunoprecipitation assay-phenylmethylsulfonyl fluoride (RIPA-PMSF) solution from harvested cells and quantified by the bicinchoninic acid (BCA) assay (Beyotime, Beijing, China). 40 μg of protein were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Next, the membranes were blocked with 5% non-fat milk at room temperature for 2 h and incubated at overnight 4°C with the primary antibodies against TopBP1 (1:1000, Abcam, Cambridge, MA, USA) and β-actin (1:2000, Abcam,

Cambridge, MA, USA). The membranes were then incubated with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 1.5 h at room temperature and visualized using enhanced-chemiluminescence (ECL) detection reagent.

Immunohistochemistry

4 μm thick sections were cut from paraffin-embedded specimens, deparaffinized with xylene and microwaved (20 min) in 0.01 mol/L citrate buffer (pH 6.0). Then the sections were treated with 0.3% hydrogen peroxide following washes with phosphate-buffered saline (PBS). The sections were incubated with TopBP1 antibody (1:100, Abcam, Cambridge, MA, USA) at 4°C overnight and polyclonal goat anti-rabbit IgG (1:250, Abcam, Cambridge, MA, USA) at 37°C for 1 h. Following washes with phosphate buffered saline (PBS), diaminobenzidine was added to the slides for 10 min and sections were counterstained with Harris hematoxylin. For negative controls, primary TopBP1 antibody was replaced with PBS.

CCK-8 Assay

Cell viability was tested by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay. All cell lines were seeded into 96-well plates at a density of 3×10^3 cells/well and cultured at 37°C. 10% CCK-8 reagent solution was added to each well for 1.5 h and measured at the absorbance of 450 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

For the colony formation assay, control and transfected cells were seeded at 400 per well into 6-well plates and cultured for 10-14 days until colonies were formed. These clones were fixed with paraformaldehyde and stained with 0.1% crystal violet. Finally, the number of colonies (at least 50 cells) was counted under the microscope.

Cell Apoptosis and Cell Cycle Assay

Apoptosis of OS cells was determined by flow cytometry using an Annexin V-APC and 7-aminoactinomycin D (7-AAD) (KeyGen Biotech, Nanjing, China). The number of apoptotic cells was analyzed using Flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA).

For cell cycle analysis, cells were fixed with ice-cold 70% ethanol overnight and stained with 50 μg/ml propidium iodide (PI) in the presence of RNase A at 37°C for 0.5 h. Then the DNA content was analyzed using Flow cytometer (FACSCalibur).

Statistical Analysis

SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses which were presented as mean±SD (standard deviation). The relation between TopBP1 expression and clinicopathological parameters was assessed by Pearson's x^2 test. Survival curves were depicted using the Kaplan-Meier survival model and survival probabilities were compared by the log-rank test. Cox proportional hazard regression models were employed to identify prognostic factors affecting survival of patients. Two-tailed Student's *t*-test was used to assess the differences between groups and $p<0.05$ was considered to be significant.

Results

TopBP1 Expression in Sarcoma Patients and OS Cell Lines

To determine whether TopBP1 was differentially expressed between sarcoma and normal tissues, we examined TopBP1 mRNA expression by analyzing the Oncomine database. A total of eight analyses met the inclusion criteria, but five were finally excluded due to the small sample sizes (n<10). Although no data regarding TopBP1 in OS were available, our analysis revealed that mRNA expression of TopBP1 was significantly elevated in other sarcoma types including myxofibrosarcoma, pleomorphic liposarcoma and leiomyosarcoma compared with normal adipose tissues (Figure 1A-C).

To investigate the TopBP1 expression in OS, qRT-PCR and Western blot were performed on OS cell lines. The results showed that TopBP1 expression levels were remarkably higher in all OS cell lines compared with the normal osteoblast line hFOB1.19 (Figure 1D-E). Among OS cell lines above, the 143B and SaoS2 cell lines were observed to have higher mRNA and protein expression of TopBP1 than other OS cell lines.

Knockdown of TopBP1 Inhibits Osteosarcoma Cell Proliferation

To explore the molecular function of TopBP1 in OS, two OS cell lines, 143B and SaoS2, which showed higher TopBP1 mRNA and protein level, were transfected with siRNA against TopBP1 (si-TopBP1) or scrambled control siRNA (si-scramble). The knockdown efficacy of siRNA was confirmed by Western blot at 48 h post-transfection. As shown in Figure 2A, si-TopBP1 markedly decreased the expression of TopBP1 in both OS cells.

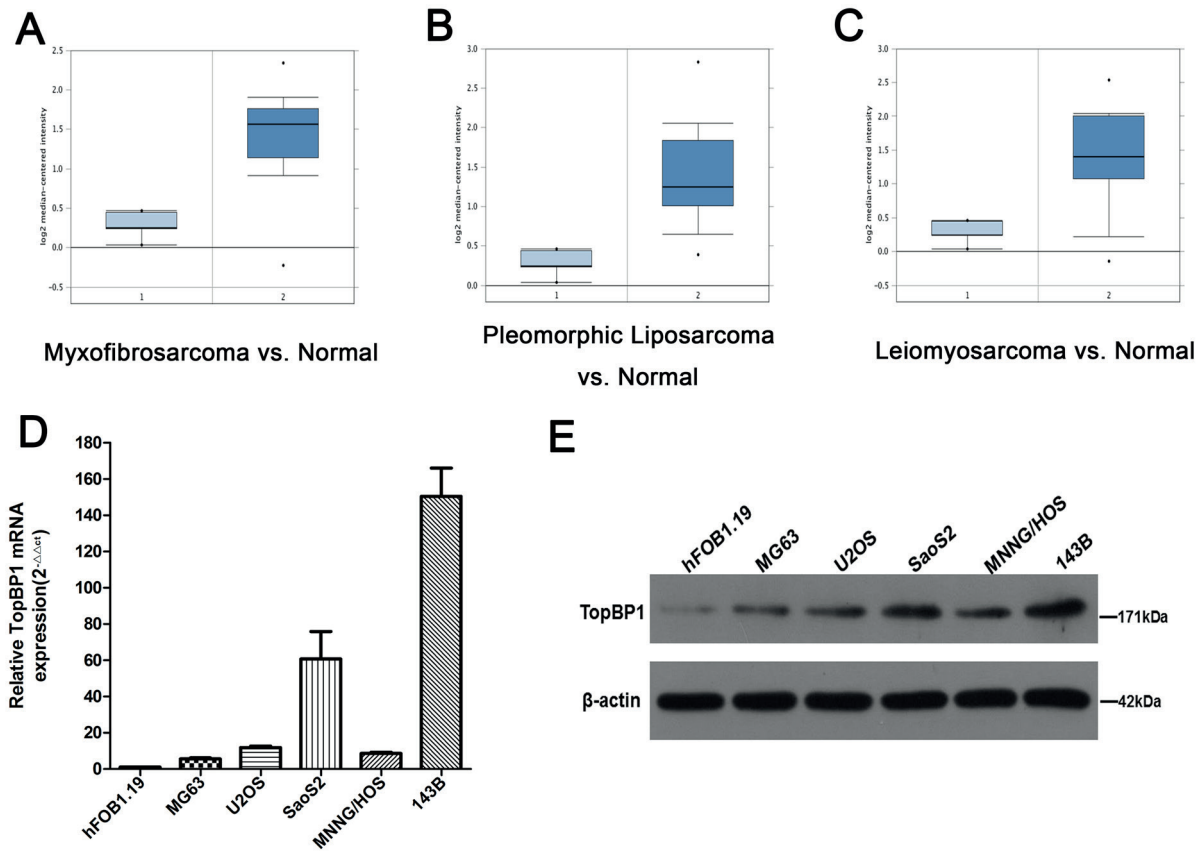

Figure 1. TopBP1 is overexpressed in sarcoma tissues and OS cell lines. **A**-**C**, TopBP1 mRNA was significantly elevated in myxofibrosarcoma (**A**), pleomorphic liposarcoma (**B**), leiomyosarcoma (**C**) than paired normal tissues. Data were collected and analyzed via the Oncomine database. **D**, qRT-PCR analysis showing TopBP1 mRNA expression in hFOB1.19 osteoblast and five OS cell lines. **E**, TopBP1 expression in hFOB1.19 osteoblast and five OS cell lines detected by Western blot.

Following CCK-8 assay was used to assess the effect of TopBP1 knockdown on cell proliferation. As shown in Figure 2B-C, the proliferation rate was not significantly different between the control cells and cells transfected with scramble siRNA, while TopBP1 knockdown significantly decreased the proliferation rate of 143B and SaoS2 cells after day 2.

We then evaluated the effect of TopBP1 silencing on OS cell growth by colony formation assay. As shown in Figure 2D-E, cells transfected with si-TopBP1 formed significantly fewer colonies compared with the control cells, indicating that silencing TopBP1 caused a decreased proliferation ability of OS cells.

Knockdown of TopBP1 Induced Cell Apoptosis and G1-Phase Arrest in Osteosarcoma Cells

To assess the effect of TopBP1 on apoptosis, Annexin V-APC/7-AAD staining of OS cells (143B and SaoS2) was conducted. Flow cytometry revealed that both cells transfected with si-TopBP1 RNA showed significantly higher apoptosis rates than the control cells (Figure 3A-B).

We next evaluated the effect of TopBP1 silencing on cell cycle distribution in OS cell lines. As depicted in Figure 3C-D, si-TopBP1 transfected 143B and SaoS2 cells showed increased percentage of G1 phase compared with the control cells, suggesting TopBP1 could cause G1-phase arrest in OS cells.

TopBP1 is Implicated in Modulating Chemoresistance in OS Cell Lines

Chemoresistance represents a major cause of poor prognosis for OS patients. To explore whether TopBP1 plays a role in modulating chemoresistance, CCK-8 assay was used to examine the effect of TopBP1 on OS cells to doxorubicin and cisplatin, two main drugs in OS chemothera-

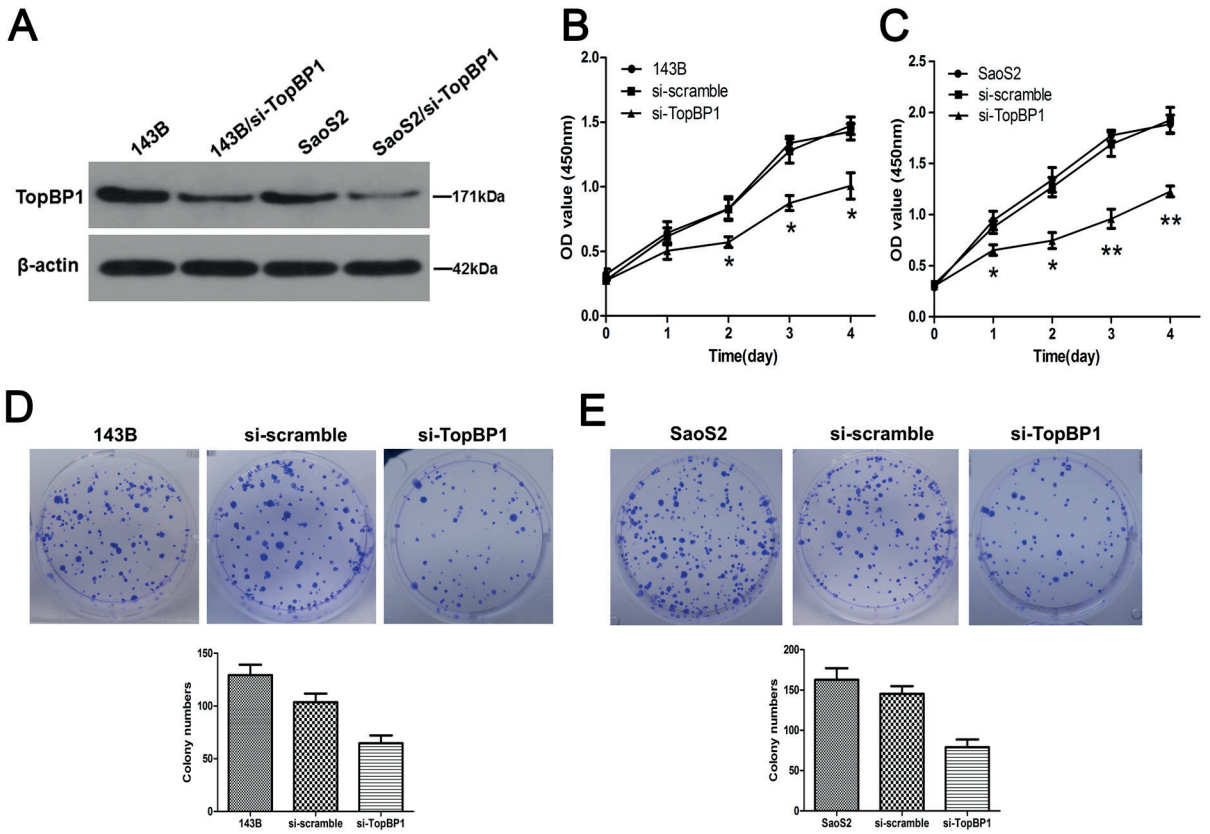

Figure 2. Silencing of TopBP1 inhibits cell proliferation and colony formation ability of OS cells. **A**, Inhibition of TopBP1 expression in OS cells by siRNA. siRNA was employed to knock down TopBP1 expression in 143B and SaoS2 cells. **B-C**, Effect of TopBP1 silencing on proliferative capacities in 143B and SaoS2 cells. Control and transfected OS cells were seeded at 3000 per well in 96-well plates and cell viability at indicated time points was measured by CCK-8 assays. ***D-E***, Impact of TopBP1 knockdown on colony formation ability. Data representative of three independent experiments are shown as mean ± SD . *$p < 0.05$, ** $p < 0.01$.

py. We observed that TopBP1 silencing sensitized 143B and SaoS2 cells to doxorubicin compared with the control one in a dose-dependent manner (all $p<0.05$, Figure 4A-B). Similarly, knockdown of TopBP1 also significantly decreased the IC50 of both 143B and SaoS2 cells to cisplatin (all $p<0.05$, Figure 4C-D).

Prognostic Significance of TopBP1 in OS Patients

The correlation between TopBP1 expression and clinicopathologic factors of OS patients was listed in Table I. TopBP1 expression was observed to be significantly associated with local recurrence ($p=0.002$) and necrosis rate ($p=0.014$), but not with age at diagnosis, gender, tumor location, lung metastasis and TNM stage (all $p>0.05$).

Kaplan-Meier survival analysis was used to explore the impact of TopBP1 expression on patient survival. As indicated in Figure 5A-B, patients with elevated TopBP1 expression had a worse overall survival ($p=0.0028$, Figure 5A) and disease-free survival (DFS), ($p=0.0024$, Figure 5B) than those with low TopBP1 expression. Univariate analysis also indicated that TopBP1 expression, lung metastasis, local recurrence, necrosis rate and TNM stage were significantly associated with overall survival and DFS (all $p<0.05$, Table II). Nevertheless, multivariate analysis failed to indicate TopBP1 as an independent prognostic factor affecting overall survival and DFS of OS (data not shown).

Discussion

OS is a rapidly proliferative and highly invasive tumor with heterogeneous prognosis. Al-

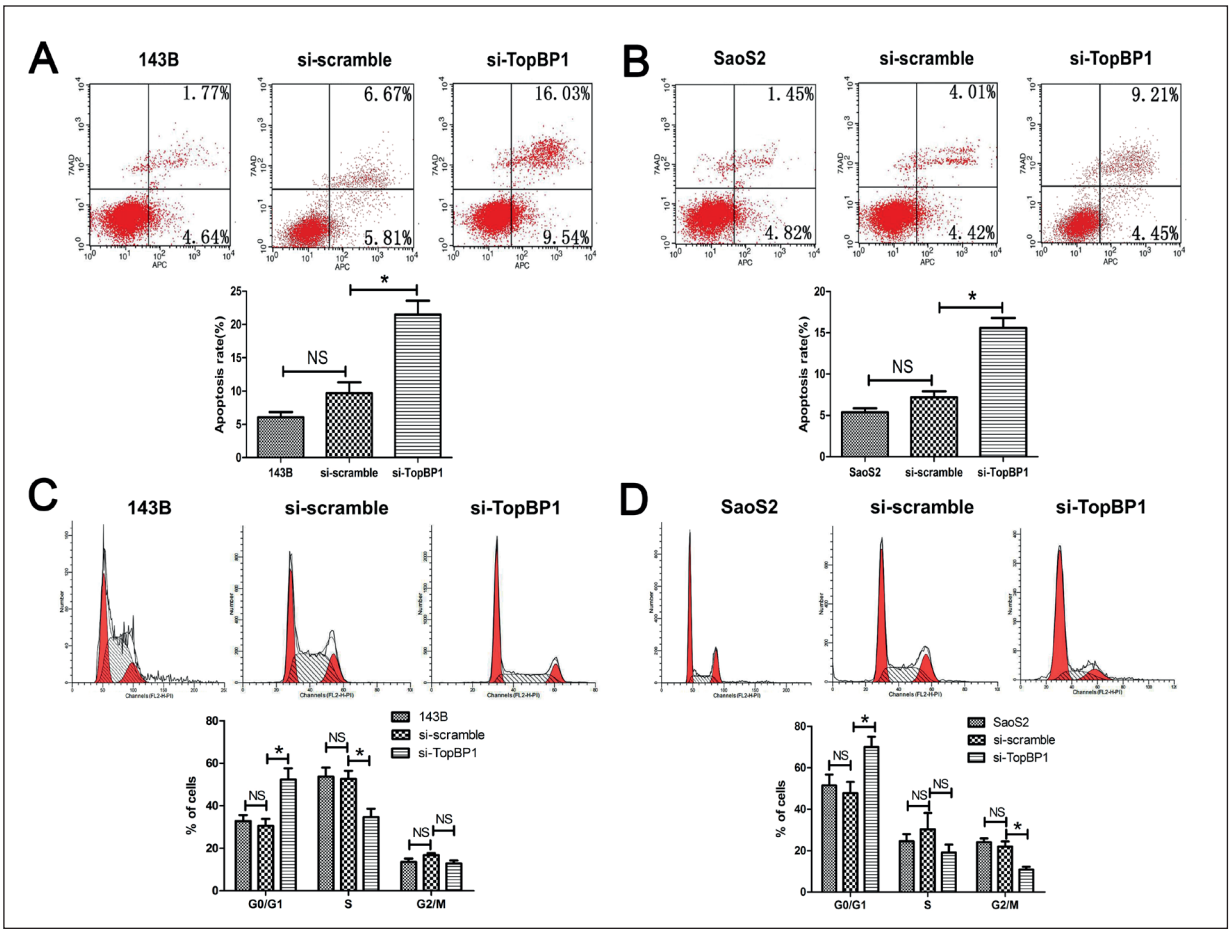

Figure 3. Decreased TopBP1 expression induces apoptosis and G0/G1 arrest in OS cells. ***A-B***, Silencing of TopBP1 cells resulted in a dramatic increase of apoptosis in 143B and SaoS2 cells. Percentage of apoptotic cells was counted via flow cytometry analysis with 7AAD/APC staining. ***C-D***, Silencing of TopBP1 in 143B and SaoS2 cells caused cell arrest in G1 phase. Cells were stained with PI and then the cycle distribution was determined by flow cytometry.

though OS treatment has achieved encouraging advances in the past decades, chemoresistance still represents a hindrance to successful treatment and the mechanisms of tumorigenesis and progression in OS remain elusive[3,15]. There is an urgent need to find novel therapeutic targets and reliable molecular biomarkers as prognostic indicators. TopBP1 protein displays similarities to BRCA1 in structure and function, and has been demonstrated to play a role in regulating DNA damage response, transcription, replication checkpoint control and transcription[16,17]. It has been reported that TopBP1 knockdown not only enhanced the doxorubicin chemosensitivity in non-small cell lung cancer and radiosensitivity in radio-resistant lung cancer cells but also prolonged the survival time after radiation therapy[12,18]. Liu et al[17] also reported that TopBP1 overexpression was observed in breast cancer tissues and correlated with shorter overall survival. However, there are limited available data about the role of TopBP1 in OS and whether TopBP1 might also be involved in affecting the malignant phenotypes of OS remains unknown.

In this present work, we firstly detected TopBP1 expression in sarcomas using Oncomine, a publicly available database that has been widely used to analyze gene expression. Although no data regarding TopBP1 expression in OS patients were available, we found that TopBP1 is upregulated in other sarcomas including myxofibrosarcoma, liposarcoma and leiomyosarcoma. Following qRT-PCR and Western-blot analysis also confirmed that TopBP1 expression was higher in all OS cell

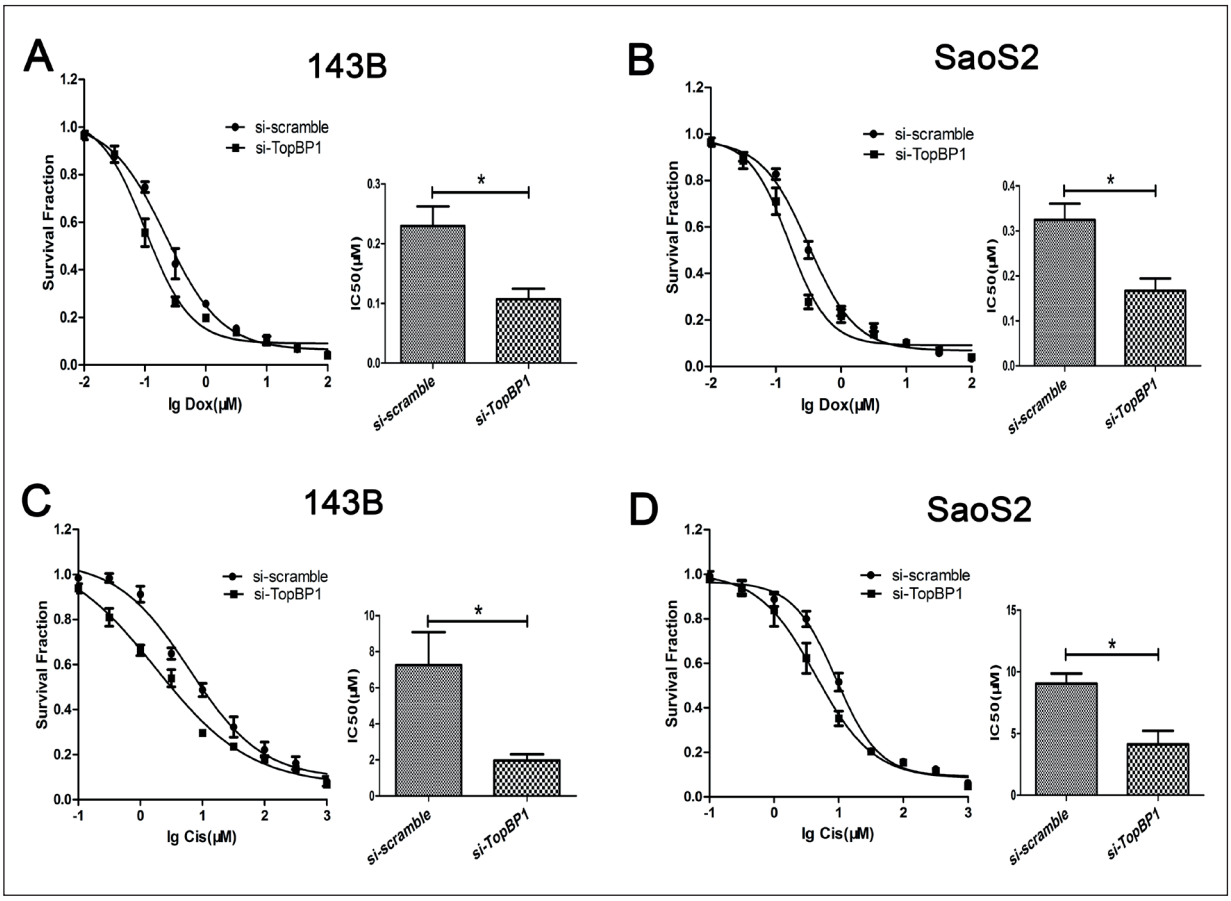

Figure 4. TopBP1 is implicated in regulating chemoresistance to both doxorubicin and cisplatin in OS cell lines. **A-B**, Dose response curves of doxorubicin in 143B (**A**) and SaoS2 (**B**) cells transfected with scramble control or si-TopBP1 RNA. **C-D**, Dose response curves of cisplatin in 143B (**C**) and SaoS2 (**D**) cells transfected with scramble control or si-TopBP1 RNA. Cellular viability was measured by the CCK-8 assay after incubating with increasing doses of doxorubicin or cisplatin for 48 h. Data from three independent experiments are shown as mean ±SD.

lines compared with the normal osteoblast line. These results prompted us to further explore the potential connection between TopBP1 expression and biological characteristics of OS cells. RNAi strategy was then employed to silence endogenous TopBP1 expression in OS cells. Following *in vitro* experiments demonstrated that TopBP1 knockdown significantly suppressed proliferation and colony formation of 143B and SaoS2 cells. We also verified that silence of TopBP1 significantly induces apoptosis of OS cells and increases the number of G0/G1 phase cells, which is consistent with the report that TopBP1 can inhibit E2F1- and P53- mediated cell apoptosis as well as P53-mediated G1 arrest[19-21]. However, the precise underlying mechanisms by which TopBP1 silencing causes inhibition of OS progression need to be further studied.

OS therapy often fails due to the development of chemoresistance, which has been attributed to mechanisms including dysregulation of ABC membrane transporters, apoptosis inhibition, enhanced DNA damage repair, detoxification in cell[22]. As TopBP1 affects the malignant phenotypes of OS, the suspicion of its influence on chemoresistance was proposed. Our paper demonstrated that TopBP1 knockdown increased sensitivity to doxorubicin and cisplatin, suggesting the development of molecular inhibitors against TopBP1 could be a potential strategy to increase the chemotherapeutic effect of OS. Additional OS cell lines should be used to further validate the effect of TopBP1 on chemoresistance of OS cells. Furthermore, our clinical investigations further confirmed that high TopBP1 expression had significantly lower overall survival and disease-free survival through the

Table I. Correlation between TopBP1 expression and clinicopathologic factors of OS patients.

Variable	Total	TopBP1 expression		x^2	*p*-value
	(n=117)	Negative	Positive		
Age (years)				2.877	0.09
≤18	74	31	43		
>18	43	25	18		
Gender				0.297	0.586
Male	74	34	40		
Female	43	22	21		
Tumor location				0.623	0.732
Femur	57	29	28		
Tibia	44	19	25		
Other sites	16	8	8		
Lung metastasis				2.59	0.108
Yes	31	11	20		
No	86	45	41		
TNM Stage				1.567	0.211
I,II	64	34	30		
III,IV	53	22	31		
Local recurrence				9.529	**0.002**
Yes	55	18	37		
No	62	38	24		
Necrosis rate				6.049	**0.014**
Low (< 90%)	51	31	20		
High (≥ 90%)	66	25	41		

Abbreviation: OS: osteosarcoma; TNM Stage: staging system for solid tumors. Significant values are indicated in bold.

Kaplan-Meier analysis, suggesting that TopBP1 expression in OS tissues may help predicting the postoperative tumor progression. This is the first study exploring the prognostic value of TopBP1 in OS patients and the result is consistent with studies in other tumors. However, several limitations such as relatively small study cohort and non-standardized experimental conditions may influence the evaluation of TopBP1's clinical value. Large multicenter clinical investigations enrolling more patient samples are needed to further verify the function of TopBP1 in clinical prognosis.

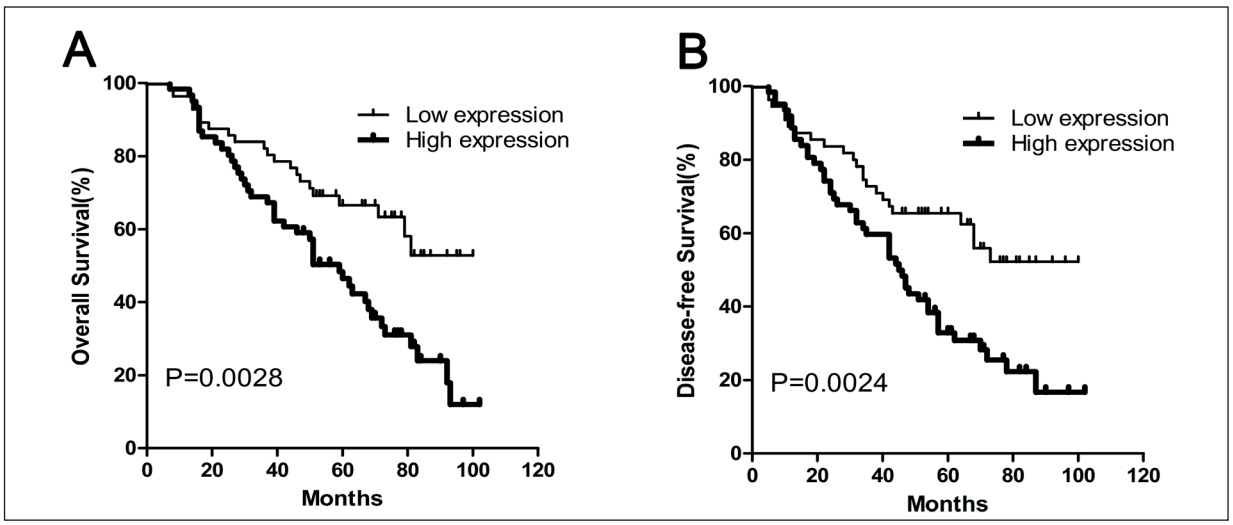

Figure 5. Kaplan-Meier curves showing the survival of osteosarcoma patients with different TopBP1 expression level. **A**, Overall survival curves stratified by TopBP1 expression in OS patients. **B**, Disease-free survival curves stratified by TopBP1 expression in OS patients.

Table II. Univariate analysis for prognostic factors of overall survival and disease-free survival.

	Overall survival			Disease-free survival		
Variable	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	1.03	0.581-1.828	0.918	1.123	0.661-1.907	0.668
Gender	0.889	0.533-1.484	0.652	0.932	0.566-1.535	0.783
Tumor Location	1.234	0.873-1.744	0.234	1.196	0.854-1.675	0.297
TopBP1 Expression	2.163	1.283-3.647	**0.004**	2.231	1.373-3.627	**0.00**1
Lung metastasis	5.128	3.070-8.566	**<0.001**	5.429	3.270-9.013	**<0.001**
Local Recurrence	11.281	5.821-21.863	**<0.001**	11.046	5.915-20.626	**<0.001**
Necrosis Rate	3.757	2.097-6.732	**<0.001**	3.511	2.020-6.101	**<0.001**
TNM stage	3.354	1.995-5.639	**<0.001**	3.027	1.849-4.954	**<0.001**

Abbreviation: HR, hazard ratio; CI, confidence interval; TNM Stage, staging system for solid tumors. Significant values are indicated in bold.

Conclusions

We combined experimental and clinical studies to explore the role of TopBP1 in OS and found that TopBP1 is significantly upregulated in OS cells and correlated with worse overall survival and DFS. Knockdown of TopBP1 suppresses cell proliferation, clonogenic ability and chemoresistance, and promotes apoptosis of OS cells. Our findings suggest a role for TopBP1 in the regulation of OS development and offer a novel target to potentially improve the chemosensitivity of OS.

Conflict of Interest

The authors declare no conflicts of interests.

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