

Dysregulation of Zinc Finger Protein 395 Contributes to the Pathogenesis of Chondrosarcoma

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Introduction: The transcription factor zinc finger protein 395 (ZNF395) is involved in several cellular responses and tumorigenesis. However, the potential role and clinical significance of ZNF395 in chondrosarcoma are not well investigated. This study determines the expression profile, prognostic value and biological function of ZNF395 in human chondrosarcoma.

Methods: The mRNA and protein expressions of ZNF395 in fresh chondrosarcomas and the matched adjacent non-tumor tissues were assessed using real-time PCR and immunoblotting, respectively. The protein expression of ZNF395 in chondrosarcoma specimens was evaluated by immunohistochemistry, and the relationships among its protein level, clinicopathological parameters and prognosis were further detected. Cell viability, colony formation, migration, invasion and apoptosis assay were evaluated in chondrosarcoma cells with depletion of ZNF395.

Results: The mRNA and protein expressions of ZNF395 in chondrosarcoma tissues were significantly higher than those in the matched adjacent non-tumor tissues and benign cartilage tumors. Clinical analysis displayed that ZNF395 was expressed at higher levels in chondrosarcoma patients with higher histological grade and advanced MSTs stage. Furthermore, we demonstrated that high expression of ZNF395 correlated with a worse overall survival of chondrosarcoma patients. Multivariate Cox regression analysis indicated that ZNF395 was an independent prognostic marker in chondrosarcoma patients. Functional studies revealed that depletion of ZNF395 markedly inhibited cell viability, colony formation, migration and invasion, and promoted apoptosis in chondrosarcoma.

Conclusion: These findings suggest that dysregulation of ZNF395 contributes to chondrosarcoma development, and ZNF395 may act as a potent oncogene and serve as a independently prognostic factor, highlight the potential of ZNF395 as a novel biomarker and therapeutic target for chondrosarcoma.

Keywords: ZNF395, chondrosarcoma development, cell growth, prognostic biomarker, therapeutic target

Introduction

Chondrosarcoma (CS) is the second most common primary bone malignancy, constituting a heterogeneous group of primary cartilaginous neoplasms. The vast majority of chondrosarcomas is conventional chondrosarcoma characterized by a relatively high recurrence because of resistance to radiotherapy and chemotherapy.¹ In recent years, deep explorations of the molecular mechanisms implicated in chondrosarcoma have presented the theoretical basis for its biological behaviors.² Accordingly, further investigations of the relationships between novel genes and the pathogenesis of chondrosarcoma have important theoretical

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significance for designing molecular targeted drugs and improving chondrosarcoma management.²

Zinc finger proteins are generally recognized as DNA-binding transcription factors and function in many biological processes such as development, differentiation, metabolism and apoptosis, and also involved in the pathogenesis and progression of human cancers.³ The Kruppel C2H2-type zinc finger motif has been found to be present in many transcription factors recognizing specific sequences of DNA.^{4,5} Belonging to the C2H2 zinc finger protein family, zinc finger protein 395 (ZNF395) is ubiquitously expressed and identified as a common transcription factor involved in several cellular responses and carcinogenesis.⁵ Many researchers have revealed that ZNF395 is frequently overexpressed in human cancers including glioblastoma, kidney cancer, osteosarcoma, Ewing sarcoma, neuroblastoma, and exerts an oncogenic role in carcinogenesis.^{6–16} On the other hand, ZNF395 is also found to have tumor suppressive activities in some cancer cells such as liver cancer cells and primary breast tumors.^{17,18} Therefore, further studies are required to resolve these inconsistent results.

To address the underlying role of ZNF395 in chondrosarcoma, we initially determined the expression profile and clinical significance of ZNF395, and further explored its biological functions in chondrosarcoma development. Our findings demonstrated that ZNF395 expression was remarkably overexpressed in chondrosarcoma specimens and significantly correlated with high-grade chondrosarcoma and poor prognosis. Moreover, ZNF395 was recognized as an independent prognostic factor for the overall survival of chondrosarcoma patients. Depletion of ZNF395 inhibited chondrosarcoma cell growth through induction of apoptosis *in vitro*. These findings highlight the potential of ZNF395 as a novel biomarker and therapeutic target for chondrosarcoma.

Methods

Clinical Samples and Chondrosarcoma Cell Line

Clinical samples including fresh chondrosarcoma tissues, the paired adjacent non-tumor tissues, conventional chondrosarcomas and benign cartilage tumors were enrolled as described in our previous study.¹⁹ Clinical clinicopathological information and follow-up data were reported previously, and signed informed consent for sample collection and analysis were obtained from all patients and was in full compliance with

national legislation and the ethical standards as described previously (IRB00001052-08044).^{20,21} Human chondrosarcoma SW1353 cell line was purchased from the American Type Culture Collection (Bethesda, MD, USA) and maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) and cultured in a humidified cell incubator with 5% CO₂ at 37°C.

Real-Time qRT-PCR

The primers for ZNF395 were followed as Forward: 5'-CGAAAAAAGAAAGAACTCTGTG-3'; Reverse: 5'-CTGTGTCCCCCAGATGGAG-3' as described previously.²² The PCR amplification for the quantification of the ZNF395 mRNAs was performed using the SYBR green mix (Applied Biosystems) with a 7500 Fast Real-Time PCR System (Applied Biosystems) as described in our previous studies.²³

Immunoblotting

The following primary antibodies were used in the immunoblotting: ZNF395 (Proteintech, 11759-1-AP, 1:1000) and β -actin (Sigma-Aldrich, A5316, 1:1000). An HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Sigma) was used at a 1:5000 dilution and detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore) as described in our previous study.²³

Immunohistochemistry and Evaluation of Staining

Detailed experimental protocols have been described previously.^{19,20} Typically, after antigen retrieval, ZNF395 (Proteintech, 13149-1-AP; 1:100) immunoreactivities were detected using the EnVision+, Peroxidase system (DAKO Diagnostics, Denmark). The slides were incubated with DAB substrate (DAKO Diagnostics, Denmark) and counterstained with hematoxylin. The percentage of positive tumor cells was graded as per the following criteria: 0, less than 10%; 1, 10–25%; 2, 26–50%; 3, more than 50%. Nucleus immunoreactivity of ZNF395 was assessed as follows: (1) a low expression level, recognized as scores of 0 for no staining and 1+ for weak staining; and (2) a high expression level, recognized as a score of 2+ for moderate staining and 3+ for strong staining. ZNF395 nucleus immunoreactivity was considered as either low (score 0–1) or high (scores 2 to 3).

siRNA Interference and Transient Transfection

Small interfering RNAs (siRNAs) targeting ZNF395 were obtained as a pool of four annealed double stranded RNA oligonucleotides from Dharmacon (siZNF395: M-020387 and siControl: D-0012061420). Cells were seeded in six wells and transfected with 50pmol siRNA using Lipofectamine RNAiMax (Invitrogen) one day later. Transient transfection was performed using the Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

Cell Viability and Clonogenic Assays

Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) for cell viability, and clonogenic formation assays were conducted as described previously.^{20,23} Briefly, SW1353 cells were seeded into 96-well plates at 5000 cells per well for 24 hours and assessed using CCK-8 at the indicated time. For clonogenic assay, after treatment, the number of viable cell colonies was determined after either 10 days SW1353 cells post inoculation of 150 cells per well in triplicate in 12-well plates. Colonies were counted after methanol/acetone (1:1) fixation and Gentian Violet staining according to the previous experimental protocols.²⁰

Cell Migration and Invasion Assays

Cell migration was detected using a modified two chamber transwell migration assay. The number of invasive cells was determined by counting the leucocrystal violet-stained cells. For the cell invasion assay, cells that had migrated across the membrane were counted in five random visual fields using a light microscope as described in our previous studies.^{21,23}

Detection of Cell Apoptosis and Caspase-3 Activity Assay

After treatment, cells were resuspended in 100µL of binding buffer, and immediately analyzed on a FACS Calibur flow cytometer (Becton Dickinson, USA) with Cell Quest Software (BD bioscience, USA) to calculate apoptotic cells. The caspase-3 activity was evaluated using a Caspase-3/CPP32 Fluorometric Assay Kit (Biovision, USA) according to the manufacturer's instructions, as described in our previous study.²³

Statistical Analysis

All data are presented as the Mean \pm SD. The Student's *t*-test or the Pearson's Chi-square test/Fisher's exact test was

utilized to analyze difference between groups with continuous variables or categorical variables, respectively. The Kaplan-Meier method and the Log rank test were applied for overall survival with chondrosarcoma patients. Univariate and multivariate analyses of clinicopathological factors, ZNF395 level and overall survival were performed using Cox regression model. Statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA) and $P < 0.05$ was considered to be statistically significant. All reported *P* values were two-sided.

Results

Elevated Expression of ZNF395 in Chondrosarcoma Specimens

To determine the underlying role of ZNF395 in the pathogenesis of chondrosarcoma, we performed Real time PCR and immunoblotting in 6 cases of the fresh chondrosarcomas and the paired adjacent non-tumor tissues, and found that the ZNF395 mRNA levels in chondrosarcoma specimens were aberrantly elevated as compared to the matched adjacent non-tumor tissues (Figure 1A, $**P < 0.01$). We also found that the protein expression of ZNF395 was significantly higher than that in the matched adjacent non-tumor tissues as shown in Figure 1B and C, $*P < 0.05$.

To further investigate the expression profile of ZNF395 in human cartilage-forming tumors, we tested the ZNF395 protein expression by immunohistochemistry assay in a retrospective cohort of 63 cases of conventional chondrosarcomas and 17 cases of benign cartilage tumors, and revealed that the positive signal of ZNF395 was preferentially recognized at the nucleus in human cartilage tumors such as enchondroma and chondrosarcoma (Figure 2A and B). In these 63 cases, high expression of ZNF395 was detected in 41 (65.1%) cases of chondrosarcomas, whereas only 4 (23.5%) cases of benign cartilage tumors showed a high ZNF395 expression ($P = 0.005$, Table 1), suggesting that dysregulation of ZNF395 may be implicated in the pathogenesis of human chondrosarcoma.

Elevated Expression of ZNF395 Predicts a Poor Prognosis

Clinical association analysis using a Pearson's Chi-square test/Fisher's exact test revealed that elevated ZNF395 expression in chondrosarcoma tissues was significantly associated with higher histological grade ($P = 0.007$) and advanced MSTS stage ($P = 0.001$, Table 2). To determine the potential role of ZNF395

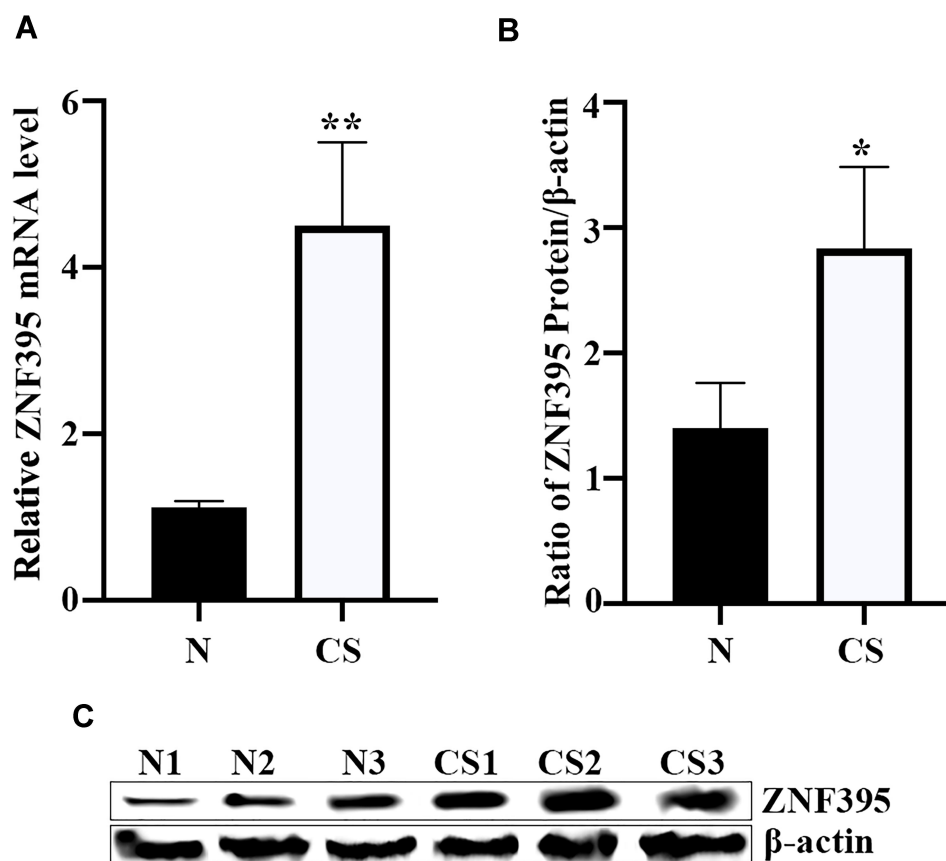


Figure 1 ZNF395 expression was significantly elevated in chondrosarcoma tissues. **(A)** ZNF395 mRNA expression was detected by Real time PCR in 6 chondrosarcoma tissues and the paired corresponding non-tumor tissues. N, the corresponding non-tumor tissues; CS, chondrosarcoma tissues. ** $P < 0.01$. **(B)** The diagram represented the statistical analysis of ZNF395 protein expression in 6 paired cases analyzed by immunoblotting. N, the corresponding non-tumor tissues; CS, chondrosarcoma tissues. * $P < 0.05$. **(C)** The protein levels of ZNF395 were analyzed by immunoblotting. To confirm the equal protein levels, the same blot was stripped and developed with anti- β -actin antibody. Three representative pairs of tissues were presented.

in predicting the prognosis of chondrosarcoma patients, nucleus immunohistostaining of ZNF395 was performed to confirm the association between ZNF395 expression and the overall survival. We had constructed Kaplan–Meier survival curves using the overall survival data to analyze cases with high or low ZNF395 expression as shown in Figure 2C. Our data indicated that the overall survival in the high ZNF395 expression group was 56.1%, compared with 86.4% in that of the low group. The median overall survival times in the high and low ZNF395 subgroups of chondrosarcoma patients were 52.6 months and 85.7 months, respectively (Figure 2C). Patients in the high ZNF395 expression group ($n=41$) had a significantly poorer prognosis than those in the low ZNF395 expression group ($n=22$) as shown in the overall survival curve (log-rank=6.640; $P=0.010$; Figure 2C). These data suggest that ZNF395 may be a prognostic marker in chondrosarcoma patients.

ZNF395 Can Be Identified as an Independent Prognostic Factor

To identify the independently prognostic factors for the overall survival of chondrosarcoma patients, we had performed Cox regression model analysis, and found that high ZNF395 level (HR, 5.735, $P=0.010$) was identified as an independent prognostic factor for poor survival of chondrosarcoma patients, in agreement with histological grade (HR, 3.217, $P=0.027$) and MSTS stage (HR, 4.571, $P=0.007$, Table 3).

Depletion of ZNF395 Inhibits Chondrosarcoma Growth and Induces Apoptosis

To determine whether ZNF395 can act as an oncogene in the pathogenesis of chondrosarcoma, we prepared siRNA oligonucleotide specifically targeting ZNF395 expression (siZNF395). As assessed by immunoblotting analysis, the

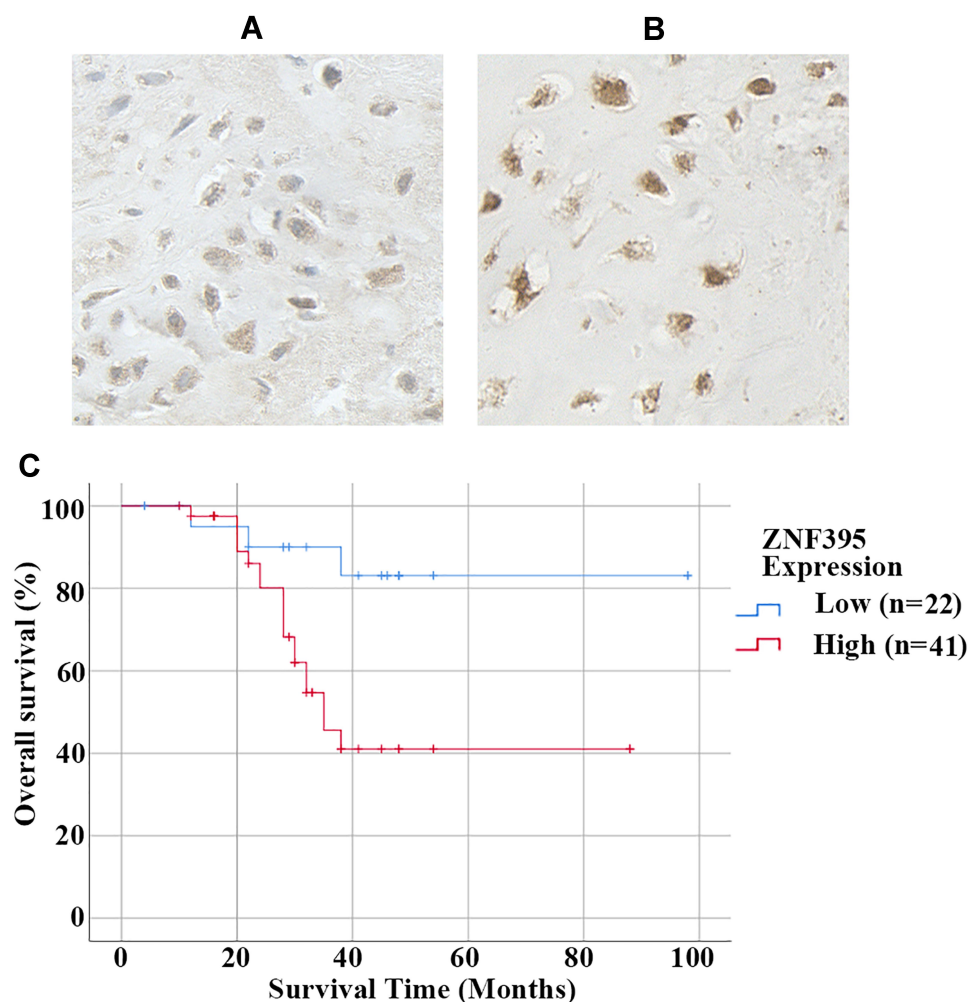


Figure 2 Elevated protein expression of ZNF395 correlated with poor prognosis in patients with chondrosarcomas. (A and B) Representative images of ZNF395 staining were presented in human cartilage tumors. Enchondroma with low nucleus immunostaining of ZNF395 immunostaining (A). High (B) nucleus immunostaining of ZNF395 were detected in chondrosarcoma tissues. (C) Prognostic values of ZNF395 protein expression in 63 patients with chondrosarcomas by Kaplan-Meier survival curves and the Log rank test. Probability of overall survival with regard to ZNF395 expression shown that high ZNF395 expression remarkably correlated with poor prognosis ($P=0.01$).

ZNF395 protein level was markedly reduced by siZNF395 as compared to that of siRNA control (siCont) in chondrosarcoma SW1353 cells (Figure 3A). Next, we tested the

effect of altering ZNF395 level on chondrosarcoma cell viability and colony formation activity by CCK-8 and clonogenic assays, respectively. As expected, depletion of ZNF395 significantly inhibited SW1353 cell viability and colony formation for both assays ($P<0.05$, Figure 3B and C). As shown in Figure 3D, the inhibition of migration and invasion of SW1353 cells transfected with siZNF395 was significantly greater than that with siCont. To clarify the potential reasons for depletion of ZNF395 mediated the growth arrest, we tested apoptosis and caspase 3 activity in these cells. Our data have unravelled that depletion of ZNF395 resulted in increased apoptosis measured by Annexin V and PI staining (Figure 3D) and cleavage of caspase 3 (Figure 3E). These findings have demonstrated that depletion of ZNF395 exerts an anti-chondrosarcoma

Table 1 Elevated Protein Expression of ZNF395 in Human Chondrosarcomas

Groups	Total No. of Cases	ZNF395 Expression		P-value
		Low	High	
Benign cartilage tumors	17	13	4	0.005*
Chondrosarcomas	63	22	41	

Notes: P values recorded are the results from Fisher's exact tests. * $P<0.05$.

Table 2 Elevated ZNF395 Expression Associated with Clinicopathological Factors in 63 Patients with Chondrosarcomas

Clinicopathological Factors	Total No. of Cases	ZNF395 Expression		P-value
		Low	High	
Gender				0.434
Male	36	11	25	
Female	27	11	16	
Age (years)				0.111
≥40	49	20	29	
<40	14	2	12	
Anatomical Location				0.578
Limb bone	21	6	15	
Axial bone	42	16	26	
Histological Grade				0.007*
Well-differentiated (I)	32	17	15	
Moderately (II)	19	4	15	
Poorly (III)	12	1	11	
MSTS stage				0.001*
I A + I B	34	18	16	
II A + II B	29	4	25	

Notes: P values recorded are the results from Chi-square tests or Fisher's exact tests. *P<0.05. MSTS stage represents Musculoskeletal Tumor Society stage.

effect by promoting both growth arrest and apoptosis, suggesting dysregulation of ZNF395 can be implicated in the pathogenesis of chondrosarcoma.

Discussion

ZNF395, as a member of the Kruppel C2H2-type zinc finger protein family, is firstly identified as a Papillomavirus binding factor (PBF).²⁴ Further studies have documented that ZNF395 exerts a transcriptional activation of interferon-stimulated genes and target genes in inflammatory microenvironment.^{7,22} Furthermore,

ZNF395 is frequently activated by hypoxia, and thus may be a direct target gene of HIF- α .^{6,25,26} Emerging evidence has demonstrated that ZNF395 is frequently overexpressed in human cancers such as glioblastoma, kidney cancer, osteosarcoma, Ewing sarcoma, neuroblastoma and clear cell renal cell carcinoma (ccRCC),^{6-16,27} suggesting ZNF395 can exert a potent oncogenic role in carcinogenesis. By contrast, ZNF395 is also suggested to have tumor suppressor activities in liver cancer cells and primary breast tumors.^{17,18} These reports have revealed that dysregulation of ZNF395 may be a frequent event in human cancers and exerts an oncogenic or tumor-suppressive role according to the specific cancer type. In regard to chondrosarcoma, the potential role of ZNF395 in the pathogenesis of chondrosarcoma remains poorly delineated. In this study, we first demonstrated that ZNF395 was significantly overexpressed in chondrosarcoma tissues as compared to the corresponding adjacent non-tumor tissues and benign cartilage tumors, supporting the notion that ZNF395 might exert an oncogenic role in chondrosarcoma development in vivo. Previously, we reported that hypoxic signaling including increased HIF-1 α or HIF-2 α levels and decreased VHL expression in chondrosarcoma tissues plays a prominent role in the development of chondrosarcoma.^{19,28,29} Furthermore, ZNF395 may be a hypoxia-induced gene, and VHL deficiency in ccRCC drives enhancer activation of ZNF395 that further potentiates tumor development.^{7,11,25} Given the ability of ZNF395 to be activated by hypoxia or VHL deficiency,^{6,11} our results combined with the previous studies suggest that ZNF395 might be activated by increased levels of HIF-1 α or HIF-2 α , or VHL deficiency in chondrosarcoma, and thereby contributed to chondrosarcoma development, representing a potential mechanism for dysregulation of ZNF395 linked to hypoxia and VHL deficiency that might contribute to the pathogenesis of

Table 3 Results of Cox Regression Analysis of the Prognostic Factors for Overall Survival in 63 Patients with Chondrosarcomas

Clinicopathological Factors	Unfavorable vs Favorable	Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
Gender	Male vs Female	1.281	0.582	1.097	0.893
Age	≥40 vs <40	1.700	0.396	2.355	0.109
Anatomical location	Axial vs Limb bone	1.298	0.588	2.077	0.210
Histological grade	Grade 3 vs Grade 1 and 2	5.186	0.000*	3.217	0.027*
MSTS stage	II A + II B vs IA + I B	3.818	0.003*	4.571	0.007*
ZNF395 level	High vs Low	4.300	0.020*	5.735	0.010*

Notes: P values recorded are the results from Cox regression analysis. *P<0.05.

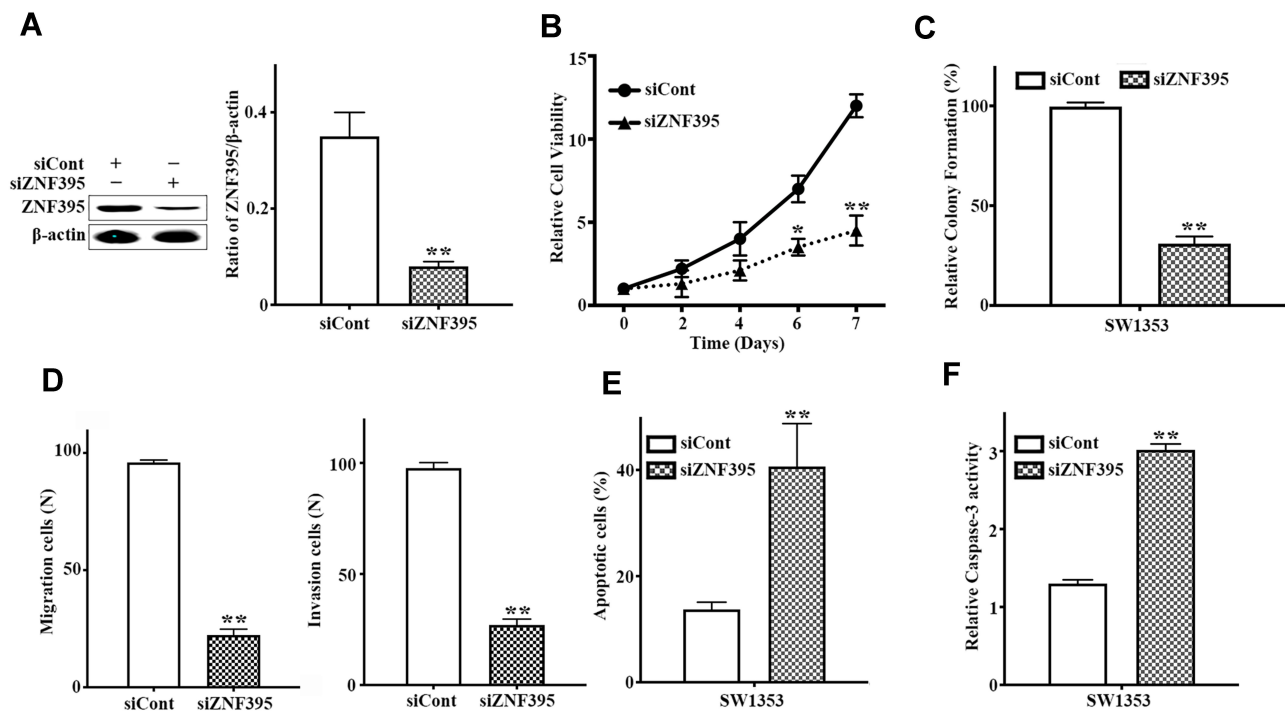


Figure 3 Depletion of ZNF395 suppressed chondrosarcoma cell growth and induced apoptosis. **(A)** ZNF395 was markedly decreased by siRNA against ZNF395. The decreased expression of ZNF395 was detected by immunoblotting from SW1353 cells transfected with control siRNA (siCont) or siRNA against ZNF395 (siZNF395) for 24 hours (Left Panel). The histograms shown the gray intensity analysis to the bands of immunoblotting (Right Panel, $**P<0.01$). **(B–D)** The inhibitory effects of siRNA against ZNF395 on cell viability, colony formation, migration, and invasion. SW1353 cells were transfected with control siRNA (siCont) or siRNA against ZNF395 (siZNF395) for 24 hours, and then incubated for the indicated time, and cell viability **(B)**, colony formation **(C)**, migration, and invasion **(D)** were detected by CCK-8, colony formation assay, and migration and invasion assay as indicated in Material and Methods. Data are plotted as the mean \pm SD ($*P<0.05$, $**P<0.01$). **(E)** Depletion of ZNF395 remarkably promoted chondrosarcoma cell apoptosis. SW1353 cells were transfected with control siRNA (siCont) or siRNA against ZNF395 (siZNF395) for 48 hours. Apoptotic cells were measured by FITC-Annexin V and PI staining followed by flow cytometry analysis. The experiment was done in triplicate, and the error bars represented standard deviations. The representative data from three independent experiments are shown. $**P<0.01$. **(F)** The activity of the pro-apoptotic caspases 3 was significantly increased after depletion of ZNF395 in SW1353 cells. SW1353 cells were treated with either siCont or siZNF395 for 24 hours. Caspase-3 activity was measured using a Caspase-3 Fluorometric Assay Kit and quantified using a POLAR-STAR fluorometer. $**P<0.01$.

chondrosarcoma. However, the relationships among ZNF395, hypoxia and VHL deficiency in chondrosarcoma development certainly merit further investigations.

Accumulated data have revealed that high expression of ZNF395 is markedly associated with a significantly poorer prognosis in the Ewing's sarcoma, osteosarcoma and neuroblastomas,^{13–16} although a previous study indicates a possible link between high ZNF395 expression and favorable prognosis in breast cancer.³⁰ Moreover, ZNF395 is identified as a potential biomarker in ccRCC.⁹ To characterize the clinical significance of ZNF395 in chondrosarcoma, we presented the first evidence that overexpression of ZNF395 was significantly correlated with histological grade and MSTS stage, rather than age, gender and anatomical location (Table 2). Importantly, our data shown that high expression of ZNF395 had significantly unfavourable impact on the overall survival of chondrosarcoma patients (Figure 2C), in agreement with the previous studies.^{13–16} Otherwise, multivariate Cox

regression model analysis found that ZNF395 was identified as an independent factor in predicting the overall survival in chondrosarcoma patients (Table 3), suggesting that ZNF395 can be identified as an independent prognostic biomarker for chondrosarcoma patients. Furthermore, several molecular markers predictive for histological grade and the prognosis of chondrosarcoma patients have been identified.³¹ Thus, our findings might help the clinician to determine the prognosis of chondrosarcoma based upon the status of ZNF395. However, further investigations for the clinical value of ZNF395 in larger cohorts of chondrosarcomas are indeed needed to conduct, which validates its clinical significance and prognosis in patients with chondrosarcomas.

Although some studies have documented that ZNF395 can be involved in the suppression of metastasis, migration and invasion,^{17,18} Sun et al have demonstrated that ZNF395 exerts an oncogenic role in ccRCC development.²⁷ To identify the underlying role of

ZNF395 in chondrosarcoma development, we had found that depletion of ZNF395 by siZNF395 can significantly suppress chondrosarcoma cell viability, colony proliferation, migration and invasion, and induce apoptosis in vitro (Figure 3), suggesting its oncogenic role in chondrosarcoma development. Furthermore, Yao et al have also proved that knockdown of ZNF395 results in increased apoptosis and the suppression of ccRCC in vivo.¹¹ In addition, several studies have delineated that ZNF395, as a tumor-associated antigen, inhibit tumor growth by activating caspase-3 and promoting apoptosis.^{12,14,24} Overall, these findings implicate ZNF395 to the proliferative and apoptosis signaling pathway. In fact, defects in apoptotic pathways are thought to contribute to the pathogenesis of human cancers, and may result in the survival of malignant tumors.³² Therefore, induction of apoptosis mediated by depletion of ZNF395 in chondrosarcoma cells to inhibit chondrosarcoma growth represents a novel treatment strategy targeting chondrosarcoma. However, the precise molecular mechanisms by which ZNF395 suppresses chondrosarcoma cell growth resulting from induction of apoptosis certainly merit further investigations.

Conclusions

Our work identifies ZNF395 is significantly overexpressed in chondrosarcoma that is remarkably associated with high-grade chondrosarcoma and poor prognosis in patients with chondrosarcoma. We have discovered that ZNF395 is recognized as an independent prognostic factor and depletion of ZNF395 suppresses chondrosarcoma growth attributed to induction of apoptosis, highlighting a novel biomarker and attractive target for chondrosarcoma treatment.

Data Sharing Statement

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Clinical samples such as benign cartilage tumors and chondrosarcomas were handled in accordance with the Declaration of Helsinki and the Human Tissue Act. The samples used in the present study were collected as discarded tissues after written informed consent was obtained from all patients, and the use of biological tissue material in this present project was covered by the ethical approvals from the Ethics Commission Peking University Third

Hospital and Tianjin Hospital as described previously (IRB00001052-08044).^{20,21}

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

All authors declared that they have no competing interest.

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