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Regular paper

NADPH oxidase 4 facilitates progression of chondrosarcoma *via* generation of reactive oxygen species

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Nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) is an enzyme that regulates reactive oxygen species (ROS) generation, and its function in the development of chondrosarcoma remains unclear. In the present study, we studied NOX4 expression in chondrosarcoma by immunochemical examination, and analyzed the role of NOX4 in viability and apoptosis of human chondrosarcoma cell line SW1353 using NOX4 siRNA or NOX4 inhibitor GKT137831. NOX4 level significantly increased in tumor compared to that in para-carcinoma sample. The levels of NOX4 were positively correlated with histological grade and Musculoskeletal Tumor Society stage of the patients. NOX4 level was significantly increased in SW1353 compared with that in chondrocytes CHON-001. Knockdown of NOX4 or inhibition of NOX4 by GKT137831 both decreased generation of ROS, and induced growth inhibition and apoptosis in SW1353, accompanied with the activation of caspases (caspase-3, caspase-8 and caspses-9), upregulation of Bax, cytochrome C(cyt-c), cleaved-PARP and down-regulation of Bcl-2. Moreover, NOX4 siRNA and GKT137831 decreased the expression of p-Akt, p-ERK and p-p65 in SW1353 cells. In an *in vivo* study, NOX4 shRNA transfected SW1353 have shown impaired growth ability compared to the SW1353 when they were injected into the nude mice. Meanwhile, GKT137831 induced growth inhibition and apoptosis in SW1353 xenograft animals, together with increased expression of Bax, cyt-*c*, cleaved-PARP, and decreased expression of Bcl-2, p-Akt, p-ERK and p-p65. NOX4 plays a positive role in the development of chondrosarcoma and could serve as a promising target against chondrosarcoma clinically.

Keywords: NADPH oxidase 4, chondrosarcoma, oxidative stress, reactive oxygen species, growth, apoptosis

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Abbreviations: DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; NOX4, Nicotinamide adenine dinucleotide phosphate oxidase 4; ROS, reactive oxygen species

INTRODUTCTION

Chondrosarcoma is a primary bone cancer with diverse morphological features and clinical behavior. Chondrosarcoma takes up about 20% of all primary malignant tumors of the bone and may occur at any age between 10 and 80 years old (MacDonald *et al.*, 2019). Although chondrosarcomas typically arise from cartilage or cartilaginous precursors within bone, they may occasionally develop within soft tissues throughout the body. At present, clinical treatment options (chemotherapy or radiation treatment) are labeled with poor response and unsatisfied effect, making a challenge to the management of chondrosarcomas (MacDonald *et al.*, 2019; Lee *et al.*, 2012; Gelderblom *et al.*, 2008). Due to absence of effective adjuvant therapy, mesenchymal malignancy has a poor prognosis.

Complicated biochemical processes involved in malignant tumor formation and development provide a lot of promising therapeutic targets and strategies. One of these strategies is to inhibit oxidative stress, as oxidative stress produced reactive oxygen species (ROS) is served as a stimulus for tumor angiogenesis (Klaunig, 2018; Ratliff *et al.*, 2016). Oxidative stress is the cellular state in which levels of ROS override antioxidant defense of the cell. ROS is an oxygen-based chemical intermediate with high reactivity. Under physiological condition, ROS level is tightly regulated through action of various scavenging and antioxidant systems to mediate the normal activity of cellular signaling pathways (Ratliff *et al.*, 2016). However, abnormally elevated ROS production that exceeds scavenging capacity of the antioxidant system has linked to pathobiology of many diseases. Many reports (Chen *et al.*, 2020; Chen *et al.*, 2020; Lee *et al.*, 2019) have supported that ROS play a positive role in the prevention of tumor programmed cell death and promotion of immortalization, metastasis, and other tumor features.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) produces superoxide anion and other ROS from molecular oxygen throughout the body. NOX transfers electrons from NADPH, they react with oxygen to form superoxide, which is usually rapidly converted to hydrogen peroxide (Ratliff *et al.*, 2016; Bedard & Krause, 2007). Therefore, NOX is a major component of oxidative stress system. At present, several members have been discovered and identified in the family of NOX, including NOX1, NOX2, NOX4, NOX5, etc. (Bedard & Krause, 2007). NOX4 is the sole NOX isoform present in human primary chondrocytes (Drevet *et al.*, 2018). Several reports confirmed the positive role of NOX4 in bone and cartilage homeostasis associated diseases (Drevet *et al.*, 2018; Wegner & Haudenschild, 2020). Moreover, NOX4 was involved in advanced glycation end-products-induced degradation of articular extracellular matrix (Lei *et al.*, 2019). However, NOX4's biological effect in the development of chondrosarcoma is still unclear. Therefore, the aim of the present study was to disclose NOX4's role in the development of chondrosarcoma, and meanwhile to evaluate effectiveness of targeted NOX4 in the prevention of chondrosarcoma.

MATERIAL AND METHODS

Clinical specimens

The present study was approved by the Ethics Committee of Anhui provincial Cancer Hospital and conducted along with the ethical standards of the Helsinki Declaration of 1975, as revised in the year 2000. A total of 40 patients with conventional chondrosarcoma were recruited into current study between 2019 and 2020 at Anhui provincial Cancer Hospital. Informed consents were obtained from all enrolled patients. None of the enrolled patients received radiotherapy or chemotherapy prior to surgery. A total of 80 samples (cancer and adjacent normal tissues) were obtained. Specimens were frozen in liquid nitrogen after resection and stored at -80°C until use.

Cells lines and culture

Human cancer cell lines

Human chondrosarcoma cell line SW1353 and chondrocytes cell line CHON-001 were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were conventionally maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and routinely cultured in a humidified incubator at 37°C supplemented with 5% CO_2.

Transfection of cells

siRNAs against NOX4 and control siRNA were purchased from Gene Pharma (Shanghai, China). SW1353 cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Briefly, the special NOX4 siRNA (40 nM) or non-binding control siRNA (40 nM) was transfected into SW1353 cells and cultured for 12h. After 12 h, the transfection medium was removed and 2 ml DMEM without FBS was added to recover cell growth for 24 h. The NOX4 siRNA sequences were sence 5'-CCAUGUGCCGAACACUCUUTT-3' and antisence 5'-AAGAGUGUUCGGCACAUGGTT-3' (Genepharma, Shanghai, China). Moreover, for stable transfection of shRNA *in vivo*, pGIPZ-lentiviral shRNA vectors targeting NOX4 gene and control vector were purchased from Open Biosystems (Thermo Fisher Scientific, Inc. Waltham, MA, USA), pGIPZ cloning vector contains a puromycin-resistant gene. Briefly, the nucleotide sequences were as follows: shNox4, 5'-GCATGGTGGTGGTGCTATTCC-3'; and shNC, 5'-TTCTCCGAACGTGTCACGT-3'. To generate lentiviral particles, HEK-293-T cells were transiently transfected with 4 mg pLKO.1 lentiviral vector (Sigma) containing shNOX4 or sh nontargeting control (Sigma) plus 4 mg of pCMVDR8.91 and 1.0 mg of pMD.2G plasmids using transfection reagent Lipofectamine 2000. SW1353 cells were transduced with lentiviral particles plus 0.4 mg/mL polybrene (Sigma) and infected overnight, and the selection of transfected cells was done by adding 0.75 mg/mL puromycin (Sigma).

Cell Viability Assay

Cell viability was analyzed using MTT assay. Briefly, 5×10^3 cells of SW1353 were seeded into 96-well plates and incubated with 1 μM cisplatin (Sigma Aldrich, St. Louis, MO, USA), 5 μM N-Acetyl-L-cysteine (NAC, Sigma Aldrich, St. Louis, MO, USA) and 1 μM GKT137831 (Med Chem Express, Shanghai, China) for 96 h. At the end of culture, DMSO was added to dissolve the formazan crystals. Absorbance was detected at a wavelength of 570 nm by a microplate reader (BioTek, Winooski, VT, USA). Percentages of cell viability (%) were determined by comparing with the untreated control.

Apoptosis assay

Cells (5×10^4/well) were treated with NOX4 siRNA for 48 h. Quantitative assessment of apoptotic cells was assessed by Annexin V/PI dual dying, as reported previously (Chen *et al.*, 2020). Briefly, at the end of the study, the cells were centrifuged at 1500 rpm for 5 min, washed twice with PBS, and resuspended in 0.5 mL binding buffer containing 1 μg/mL propidium iodide (PI) and 0.025 μg/mL annexin V-FITC (BD Biosciences, CA, USA). Finally, apoptotic cells were analyzed by using FACScan and the CellQuest program (Becton Dickinson, Lincoln Park, NJ, USA).

Measurement of caspases activities

Cells were seeded on 6-well plates (5×105 cells/well) and treated with1μM GKT137831 for 48h or transfected by siRNA NOX4 for 48h. Caspase-3, caspase-8, and caspase-9 activities were measured using commercial colorimetric assay kits (Beyotime, Haimen, China) according to the protocol provided by the manufacturer.

Measurement of intracellular ROS

Intracellular levels of ROS were determined using the fluorescent probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were treated with NOX4 siRNA for 48 h, and then were centrifuged, suspended in PBS, and loaded with 20 μM DCFH-DA at 37°C for 30 min. Fluorescence intensity of the formed DCF was analyzed by flow cytometry (Beckman Coulter, Fullerton, CA, USA). Similarly, ROS levels in the grown tumor were measured as described previously, based on the oxidation of DCFH-DA. ROS formation was quantified from a DCF standard curve, and data are expressed as pmol DCF formed/min/mg protein.

Western blot analysis

Western blotting analysis was performed to semi-quantitatively measure targeted proteins. In brief, the cellular or tissue lysates were prepared, and protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Blots were blocked with 4% BSA for 1 h at room temperature and then incubated with antibodies against NOX4 (1:1000, Abcam, Cambridge, MA, USA), Bax (1:1000, Abcam, Cambridge, MA, USA), Bcl-2 (1:1000, Abcam, Cambridge, MA, USA), cytochrome c (cyt-c, 1:1000, Abcam, Cambridge, MA, USA), cleaved poly ADP-ribose polymerase (PARP, 1:1000, Abcam, Cambridge, MA, USA), phosphorylated Akt (p-Akt, 1:1000, Abcam, Cambridge, MA, USA), Akt (Akt, 1:1500, Abcam, Cambridge, MA, USA), p-ERK (1:1000, Abcam, Cambridge, MA, USA), ERK (1:1500, Abcam, Cambridge, MA, USA), p-p65 (1:1000, Abcam, Cambridge, MA, USA), and p65 (1:1000, Abcam, Cambridge, MA, USA). After being washed three times, the blots were incubated with peroxidase-conjugated secondary antibody for 1 h at

room temperature, and finally, the bands were visualized by enhanced chemiluminescence gel imaging system, and semi-quantitatively analyzed by Image J software.

In vivo tumor xenograft study

The present animal study was approved by animals' care and use of ethics committee of Anhui provincial Cancer Hospital and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. Male BALB/c nude mice (6 weeks old) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in a temperature-controlled room with a 12 h light/12 h dark cycle and provided with autoclaved chow and water *ad libitum*. SW1353 cells or shRNA transfected SW1353 cells (5×10^6 cells in 100μl) were injected subcutaneously into the flanks of nude mice, and tumors were allowed to develop for 14 days until they reached a size of approximately 100 mm^3. Animals were divided into control group, cisplatin group (5 mg/kg, intragastric administration), GKT137831 group (50 mg/kg, intragastric administration), shRNA control group and NOX4 shRNA group. The mice were treated with vehicle or medicine for 14 days. The volume of implanted tumor of mice was measured twice a week with a caliper, using the formula (width^2×length)/2. At the end of the study, all mice were killed, and the tumors were surgically excised, weighed and stored for further use.

Immunohistochemistry detection

Immunohistochemical (IHC) staining of tissue sections were performed using the manufacturer's standard protocols (Beyotime, Haimen, China). Briefly, sections were deparaffinized, and hydrated in a graded alcohol series. The protein reacted with antibody of NOX4 overnight at 4°C. Finally, the slides were incubated for 5 min with chromogen 3,3'-diaminobenzidine tetrahydrochloride. The level of NOX4 was analyzed by a pathologist according to the positive staining area.

Statistical analysis

The data are represented as the mean ± standard deviation. Statistical analysis between groups was performed using one-way analysis of variance and Dunnett's tests (SPSS15.0, SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P<0.05$.

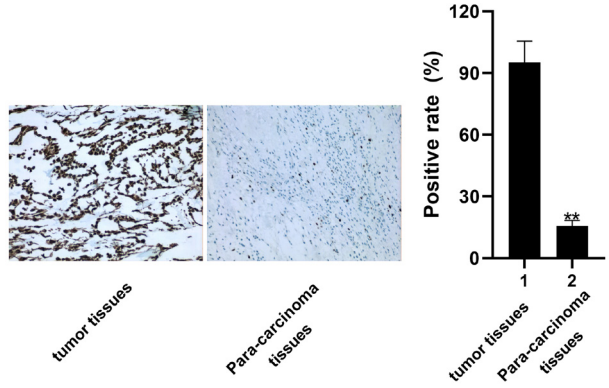

Figure 1. Representative images of NOX4 immunohistochemical staining of patients with conventional chondrosarcoma. Numerous positive cytoplasmic NOX4 staining was observed in the tumor samples, but not in the para-carcinoma samples. Results are expressed as the mean ± S.D. $^{**}P<0.01$ compared with tumor samples.

RESULTS

NOX4 overexpressed in chondrosarcoma

As shown in Table 1 and Fig. 1, 40 pairs of chondrosarcoma specimens were collected, and NOX4 expression in the tumor sites was detected and compared with that in para-carcinoma sites *via* IHC assay. The data in Fig. 1 has shown that NOX4 expression was significantly increased in the tumor tissue compared with those in the para-carcinoma ($P<0.01$). NOX4 was lightly expressed in the cytoplasm and nuclei of para-carcinoma sample, however, high staining was observed in the cytoplasm and nuclei of most tumor samples. We analyzed clinicopathologic parameters of the patients and then divided them into two groups based on the NOX4 expression. The result is summarized in Table 1. Briefly, it was interesting to find that NOX4 levels were correlated with histological grade and MSTS stage in patients.

Table 1. Association between clinicopathologic parameters and NOX4 levels in chondrosarcoma

Characteristics	Numbers		*P* value
	NOX4 high	NOX4 low	
Age (years)			
> or= 40	12	11	> 0.05
<40	8	9	
Gender			
Male	9	13	> 0.05
Female	11	7	
Anatomical Location			
Limb bone	14	10	> 0.05
Axial bone	6	10	
Histological Grade			
Well/moderate	16	7	<0.01
Poor	4	13	
MSTS Stage			
IA + IB	5	14	<0.01
IIA + IIB	15	6	

$P<0.05$ statistically significant difference; Musculoskeletal Tumor Society: MSTS

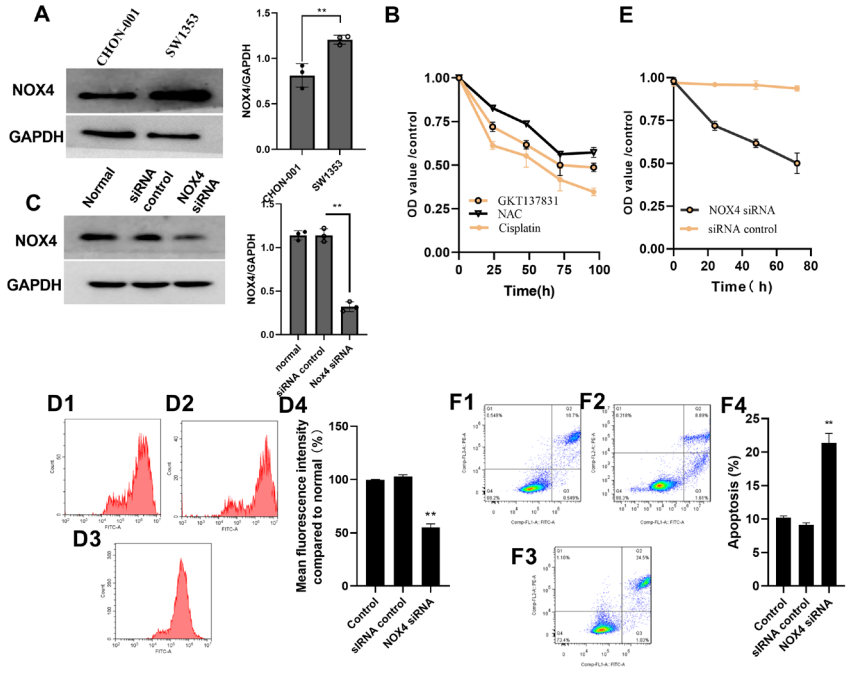

Figure 2. Depletion of NOX4 inhibits chondrosarcoma cell line SW1353 viability.
(**A**) NOX4 levels in cell line SW1353 and chondrocytes CHON-001 were evaluated by western blotting,$^{**}P<0.01$ compared with CHON-001 group; (**B**) SW1353 was treated with 5μM NAC, 1μM GKT137831 and 1μM cisplatin for 96h. The viability of cells was determined by MTT assay; (**C**) NOX4 was knockdown in SW1353 by siRNA interference; (**D**) ROS generation of SW1353 (D1), siRNA control (D2) and siRNA NOX4 (D3) was measured by FACS analysis using DCFH-DA, and results were shown in D4; (**E**) Viability of NOX4 silenced SW1353 was determined by MTT assay; (**F**) Apoptosis of NOX4 siRNA transfected cells were detected by propidium iodide/annexin V-FITC dual dying. F1: SW1353; F2: siRNA control; F3: siRNA NOX4; Results are expressed as the mean ± S.D. $^{**}P<0.01$ compared with siRNA control group.

NOX4 mediated viability and apoptosis of chondrosarcoma cell line SW1353

Expression of NOX4 was measured in chondrosarcoma cell line SW1353 and chondrocytes cell line CHON-001. Figure 2A has shown that NOX4 expression was significantly higher in SW1353 than in CHON-001 cells. We used antioxidants NAC and NOX4 specific inhibitor GKT137831 to verify the effect of NOX4 on cell viability. The data in Fig. 2B has shown that SW1353 vi-

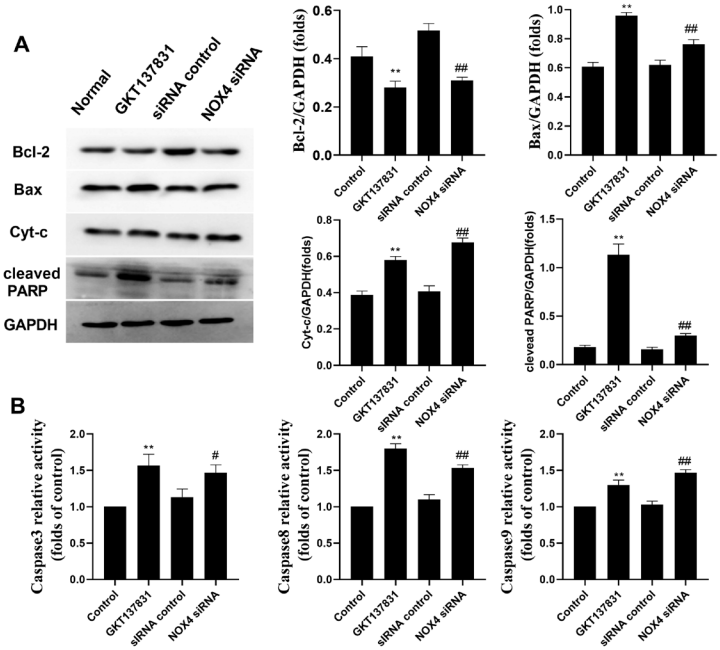

Figure 3. Depletion and inhibition of NOX4 mediates activation of Caspases and expression of apoptotic related proteins.
(**A**) SW1353 were treated with 1 μM GKT137831 or transfected by NOX4 siRNA for 48 h, and expression of apoptotic related proteins was evaluated by western blotting analysis; (**B**) caspases activities were measured by commercial kits. Results are expressed as the mean ± S.D. $^{**}P<0.01$ compared with control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with siRNA control group.

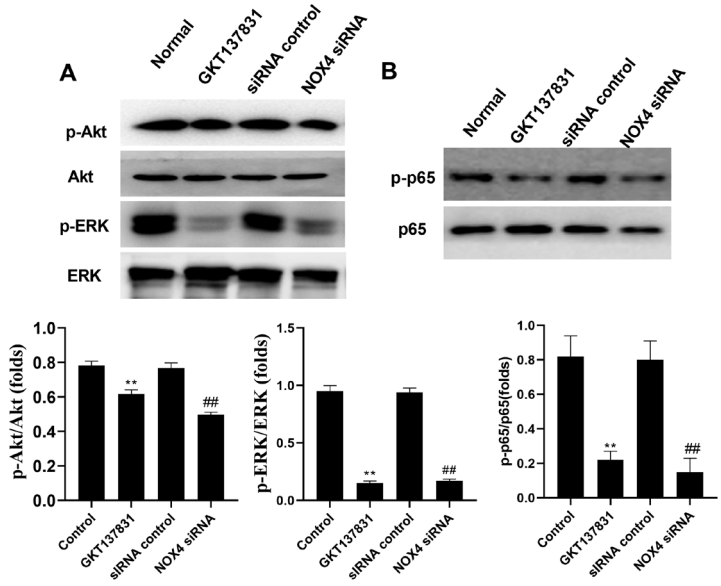

Figure 4. Depletion and inhibition of NOX4 decrease the expression of pro-survival proteins.
SW1353 was treated with 1 μM GKT137831 or transfected by NOX4 siRNA for 48 h. Pro-survival protein p-Akt, Akt, p-ERK, ERK (**A**), p-p65 and p65 (**B**) were evaluated by western blotting analysis. Results are expressed as the mean ± S.D. $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with siRNA control group.

ability was inhibited by GKT137831 as well as NAC in a time-dependent manner. To further disclose bio-function and effect of NOX4 on cells viability, NOX4 knockdown cell model was developed in SW1353, and changes in the expression of NOX4 and ROS generation were verified by western blotting and flow cytometry analysis (Fig. 2C–2D, $P<0.01$). As seen in Fig. 2E–2F, cell viability and apoptotic death in the NOX4 depletion group was significantly attenuated and enhanced, respectively, compared with the control group ($P<0.01$).

Blocked or silenced NOX4 activated apoptotic related pathway in SW1353

ROS mediates activation of apoptotic related proteins (Bedard & Krause, 2007). Therefore, we measured the change in the expression of cyt-c and Bcl-2 family proteins. As shown in Fig. 3A, NOX4 knockdown by NOX4 siRNA or functional inhibition by GKT137831 greatly increased cytosolic cyt-c, Bax and cleaved-PARP compared with that in the control group. On the other hand, treatment of the cells with GKT137831 or siRNA NOX4 decreased level of Bcl-2 ($P<0.01$). Moreover, we have observed NOX4 siRNA and GKT137831 greatly increased activity of caspases family members (caspase-3, caspase-8 and caspase-9) as well.

Blocked or silenced NOX4 inhibited activation of pro-survival proteins in SW1353

Pro-survival protein Akt, ERK and NF-κB are mediated by ROS as well (Coso *et al.*, 2012). Therefore, we further measured the change in expression of p-Akt, Akt, p-ERK, ERK, p-p65 and p65 by western blot analysis. As shown in Fig. 4A, the group that received NOX4 siRNA or GKT137831 showed decreased ratios of p-Akt/Akt and p-ERK/ERK compared with control group ($P<0.01$). Moreover, as can be seen from the results present in Fig. 4B, p-p65/p65 was markedly decreased in cells that received NOX4 siRNA or GKT137831 ($P<0.01$). Therefore, it is suggested that NOX4 mediated SW1353 apoptosis may occur through the regulation of pro-survival proteins.

Blocked or silenced NOX4 effectively inhibited growth of chondrosarcoma in vivo

On the basis of NOX4 mediated pro-apoptotic effect *in vitro*, we further verified whether blocking or silencing NOX4 could possess an anti-chondrosarcoma action. As shown in Fig. 5A–B, in mice injected with NOX4 shRNA transfected cells, the tumor size and weight were significantly smaller than that of shRNA control group ($P<0.01$). Moreover, in the mice treated with GKT137831 or positive drug Cisplatin, the tumor size and weight were significantly smaller than that of control group as well ($P<0.01$). Further *ex vivo* analysis of tumors excised from mice was conducted (Fig. 5C–D), and we confirmed that NOX4 expression and ROS production were effectively reduced by treatment with NOX4 shRNA or GKT137831 ($P<0.01$). As seen in Fig. 6, increased expression of Cyt-c, Bax, cleaved-PARP and decreased level of Bcl-2 were shown in GKT137831/NOX4shRNA groups compared with control group ($P<0.01$). Moreover, we found that p-Akt/Akt, p-ERK/ERK and p-p65/p65 ratios in GKT137831/NOX4 shRNA groups were remarkably decreased compared with control group ($P<0.01$).

DISCUSSION

In recent years, more and more investigations focus on tumor prevention based on inhibiting oxidative stress (Klaunig, 2018). Recent investigations have implicated various NOX isoforms in pathological processes of malignant tumor. For instance, it has been reported that up-regulated NOX1 expression in gallbladder cancer associated fibroblasts predicts a poor prognosis (Wang *et al.*, 2019), and NOX2 expressed in endosomes promotes cell proliferation and prostate tumour development (Harrison *et al.*, 2018). However, little is known about the role of NOX in the development of chondrosarcoma. In previous studies (Drevet *et al.*, 2018; Wegner & Haudenschild, 2020; Lei *et al.*, 2019), it has been reported that among 7 NADPH oxidases expressed in humans, NOX4 is the sole isoform present in human primary chondro-

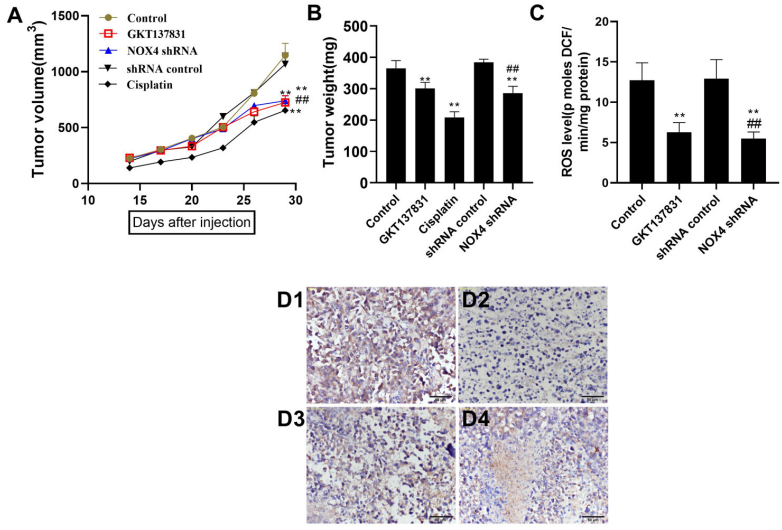

Figure 5. Depletion or inhibition of NOX4 abolishes growth of chondrosarcoma xenografts in mice.
Nude mice bearing tumor xenografts were injected intraperitoneally with vehicle or GKT137831. (**A–B**) Tumor volumes and weight were measured; (**C**) ROS levels were measured based on the oxidation of DCFH-DA to DCF in the tumor samples; (**D**) Immunostaining detection was used to examine NOX4, in which D1 represent control mice, D2 represent GKT137831 treated mice; D3 represent shRNA control mice and D4 represent NOX4 shRNA treated mice. Results are expressed as the mean ± S.D. $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with shRNA control group.

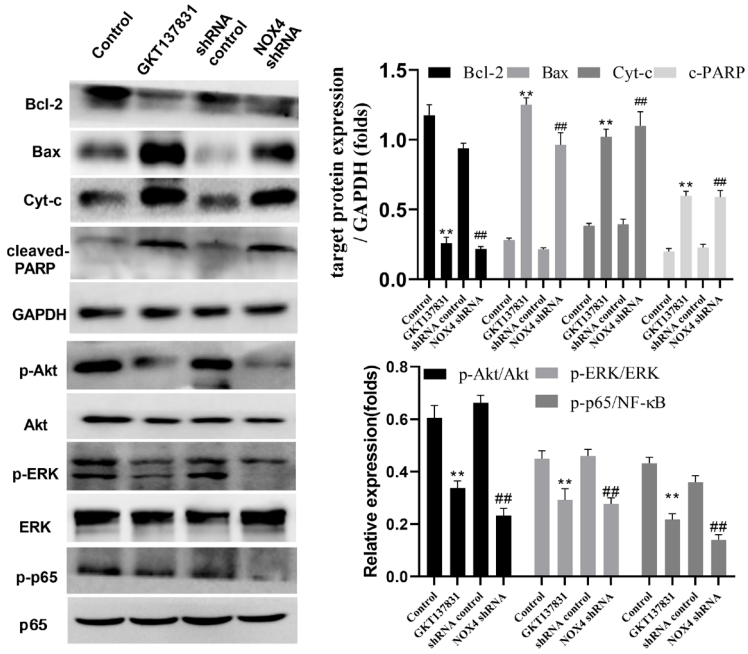

Figure 6. Depletion or inhibition of NOX4 mediates activation of apoptotic related proteins in *vivo*.
Protein in the tumor was examined by western blotting assay. Results are expressed as the mean ± S.D. $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with shRNA control group.

cytes. Moreover, NOX4 has been involved in the degradation of articular extracellular matrix. Therefore, we suspect that NOX4 plays an important biological effect in the development of chondrosarcoma.

In the present study, we collected clinical samples from chondrosarcoma patients who received surgical operation and found that the NOX4 expression significantly increased in tumor compared with those in paracarcinoma samples. To the best of our knowledge, this was the first time to semi-quantitatively analyze NOX4 in chondrosarcoma. Further study was conducted to investigate the effect of NOX4 inhibitor on viability of SW1353. In previous studies, diphenylene iodonium (DPI) has been extensively used to inhibit activity of NOX4 because of its powerful inhibition (Yao *et al.*, 2017; Hwang *et al.*, 2020). However, several studies (Rand & Li, 1993; Castro *et al.*, 2009) found that DPI is a non-selective NOX4 inhibitor with mild inhibition on nitric oxide synthase and xanthine oxidase. GKT137831 is a specific inhibitor of NOX4 with high selectivity. Therefore, we used GKT137831 to treat SW1353. In the current study, GKT137831 induced an inhibitory ef-

fect on viability of SW1353 in a time-dependent manner and has shown a similar effect to that of positive medicine cisplatin. Additionally, to identify the potential role of NOX4 in the viability of chondrosarcoma cells, we transfected SW1353 with NOX4 siRNA and then validated transfection efficiency by western blotting and ROS generation. Our results revealed that prevention of NOX4 induced a significant increase in the proliferation inhibition and apoptosis of the cells. More importantly, animal studies demonstrated that NOX4 knockdown or function inhibition by GKT137831 significantly impaired growth of the tumor. In line with previous reports regarding the role of NOX4 in the development of malignant tumors (Tang *et al*., 2018; Li *et al*., 2014), NOX4 is a promising pharmacological target for chondrosarcoma prevention.

NOX4 generated ROS has a close relationship with intrinsic apoptosis pathway (Coso *et al*., 2012). In brief, the status of intrinsic pathway is governed by interplay between pro- and anti-apoptosis members of the Bcl-2 family, including Bcl-2 and Bax. They keep cell death in check by restraining the pro-apoptotic protein Bax, which are the final arbiters of cell death. When activated, Bax triggers mitochondrial damage, allowing release of cyt-*c* and caspases activation (Franklin *et al*., 2011; Jendrossek *et al*., 2012). Here, we found that NOX4 knockdown by siNRA intervene, or function inhibited by GKT137831 significantly activate mitochondrial related apoptosis pathway, which was evidenced by increased expression of Bax, cyt-c, decreased level of Bcl-2, and activation of caspase-3, caspase-8 and caspase-9. In agreement with these observations, we noted similar results *in vivo* as well.

Some second messengers (such as protein kinases) and the third messenger are well known downstream of ROS. The first one is Akt, and it is a serine threonine kinase that mediates various biological functions, such as cell proliferation, survival, glucose metabolism, protein synthesis, genome stability, and inhibition of apoptosis in response to different growth factors and extracellular stimuli. Akt inactivates pro-apoptotic proteins (Bcl-2 antagonist of cell death and procaspase-9) to block apoptosis (Shariati & Meric-Bernstam, 2019; Downward, 2004). The second one is ERK. RAS-RAF-MEK-ERK pathway is the most well-studied of the mitogen-activated protein kinase cascades and is critical for cell proliferation, differentiation, and survival. Activation of receptor tyrosine kinases by growth factors and extracellular signals results in sequential activation of RAS, RAF, MEK, and finally ERK, which activates numerous transcription factors and facilitates oncogenesis in the case of aberrant pathway activation (Asati *et al*., 2016; Barbosa *et al*., 2021). The third one is NF-$\varkappa$B p65, and it is a ubiquitous transcription factor that mediates cytoplasmic/nuclear signaling pathway and regulates gene expression of various cytokines, cytokine receptors, which affected cell proliferation, differentiation, migration, and angiogenesis in tumor cells (Soleimani *et al*., 2020). To disclose underlying mechanism involved in the NOX4 mediated apoptosis of chondrosarcoma cells, we focused on the effect of blocking NOX4 on activity of Akt, ERK as well as NF-$\varkappa$B. Here, we showed that NOX4 knockdown or functional inhibition both induced decreased levels of p-Akt, p-ERK and p-p65 *in vivo* and *in vitro*. These results, together with impaired tumorigenic capacity of NOX4-silenced SW1353 in mice, suggest that NOX4 acts as a growth promoter in chondrosarcoma.

CONCLUSIONS

Our results suggest that NOX4 mediates proliferation and apoptosis of chondrosarcoma cells. Moreover, inhibition of NOX4 induced tumor growth may be via activation of intrinsic apoptosis pathway and regulation of pro-survival factors Akt, ERK and NF-$\varkappa$B. NOX4 is a novel and promising target for the drug development against chondrosarcoma.

Declarations

Acknowledgements. The current study did not receive any Financial Support.

Ethical Issue. The present study was approved by the Ethics Committee of Anhui Provincial Cancer Hospital and conducted along with the ethical standards of the Helsinki Declaration of 1975, as revised in the year 2000.

Conflict of interest. The authors declare that there are no conflicts of interest.

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