The role of *APOBEC3B* in chondrosarcoma

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Abstract. Chondrosarcomas rank as the third most common type of bone tumors. In the present study, we demonstrated that expression of the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3B (APOBEC3B) was higher in cancer tissues when compared to that in normal tissues. In order to further investigate the effects of APOBEC3B expression, we knocked down *APOBEC3B* expression in chondrosarcoma cells. We found that the percentage of apoptotic cells was higher in the APOBEC3B-knockdown cells than the percentage in the untransfected cells. Furthermore, we found that the reduced antitumor activity of RUNX3 was caused by APOBEC3B. Finally, we demonstrated that caspase-3, -8 and -9 activity was significantly increased in the *RUNX3*-expressing cells with APOBEC3B knockdown. In summary, our results indicate that APOBEC3B knockdown may be a useful therapy to enhance apoptosis in chondrosarcoma.

Introduction

Chondrosarcomas rank as the third most common type of bone tumors, after myelomas and osteosarcomas (1). Due to its recalcitrance to chemotherapy and radiotherapy, chondrosarcoma is primarily treated with surgery (2). After adequate resection, the 10-year survival of patients with grade I chondrosarcoma is excellent, whereas the rate is only 64% for grade II and 29% for grade III tumors (3). Thus, in recent years, several novel therapeutic approaches have been evaluated in experimental studies (3,4).

The apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3 (APOBEC3) family of proteins is a major component of the innate immunity system, acting against a variety of viruses (5,6). The APOBEC family is comprised of a series of molecules with conserved cytidine deaminase domains (CDAs), including AID, APOBEC1, APOBEC2, APOBEC3A to H and APOBEC4 (7,8). APOBEC3B expression is relatively high in many different cancer cell lines, such as breast cancer and in lymphocytes (9,10). Prostate and renal clear cell carcinomas showed statistically significant upregulation of APOBEC3B in the tumors (11). Six different cancers, breast, uterus, bladder, head and neck, and lung, show evidence of strong APOBEC3B upregulation in the majority of tumors (11).

However, the role of APOBEC3B in chondrosarcoma remains unclear. In the present study, to better understand this issue, we performed quantitative analysis on the association of APOBEC3B with the risk of developing chondrosarcoma in a Chinese Han population.

Materials and methods

Tissue samples. All chondrosarcoma and adjacent non-tumor tissue samples were obtained from the First Hospital of China Medical University from June 1993 to June 2013, following the consent of each patient. The procedure was approved by the China Medical University Ethics Committee. The study population consisted of 34 men and 18 women and the mean age was 44 years (range, 19-68 years). Twenty-three of the 52 cases were histologic grade I tumors, 15 were grade II tumors, and the remaining 14 were grade III.

Cell culture. The human chondrosarcoma cell lines, SW1353 (derived from a human grade II chondrosarcoma) (12) and OUMS-27 (derived from a human grade III chondrosarcoma) (13), and RUNX3-positive SW1353 cells (14) were stored in our laboratory and maintained in minimum essential medium (MEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin and 100 mg/ml of streptomycin) at 37˚C in a 5% (v/v) CO_2 incubator.

Plasmid and transfection. SW1353 and OUMS-27 cells were seeded in 10-cm dishes and grown overnight to 70% confluency, trypsinized and transfected with the APOBEC3B shRNA plasmid (sc-72515-SH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Colony formation assay. Cells were seeded at 200 cells/well in 24-well tissue culture plates. Plates were incubated for 3 weeks in a humidified incubator at 37˚C. Three weeks after seeding, colonies were stained with 0.05% crystal violet containing

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50% methanol and counted. The colonies were counted in 4 to 5 random fields for each of the duplicate samples by using a microscope at x100 magnification.

Measurement of apoptotic cell death. Cells were harvested 48 h after transfection, and immunostained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (Apoptosis Detection kit; KeyGen, Nanjing, China). Data analysis was performed using CellQuest software (BD Biosciences, Baltimore, MD, USA).

Transwell migration assay. Cells were plated at $2x10^5$ cells/well in 0.5 ml of serum-free medium in 24-well Matrigel-coated Transwell units with polycarbonate filters (8-μm pore size; Costar Inc., Milpitas, CA, USA). The lower chamber was loaded with 600 μl of MEM containing 10% FBS. After incubation for 24 h in normal culture conditions, the top surface of the membrane was gently scrubbed with a cotton bud and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and stained with crystal violet, and the cells that had invaded through the membrane filters were counted using a light microscope. Ten microscopic fields (x400) were randomly selected to count the cells.

Real-time PCR. Total RNA was isolated using an RNeasy Mini kit (Biomed, Beijing, China). First-strand cDNA was reverse transcribed with 1 μg of total RNA, using the Takara reverse transcription kit and oligo$(dT)_{15}$ primers (both from Takara, Dalian, China). The resultant cDNA was then used for quantitative PCR reactions. The *APOBEC3B* primers were: 5'-TAGGTGCCACCCCGAT-3' (sense) and 5'-TTGAGC ATAATCTTACTCTTGTAC-3' (antisense). The housekeeping gene, *GAPDH*, was used as the internal control for normalization of the results. The *GAPDH* primers were: 5'-AGAAGG CTGGGGCTCATTTG-3' (sense) and 5'-CGATCCACACGG AGTACTTGC-3' (antisense). Amplification of *APOBEC3B* and *GADPH* was performed with 1 cycle at 95˚C for 10 min, and 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. Calculation of the relative expression of each transcript was performed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Equal amounts (30 μg) of cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, and incubated with specific antibodies. The reaction was followed by probing with peroxidase-coupled secondary antibodies, including anti-rabbit IgG or anti-mouse IgG antibodies at dilutions ranging from 1:1,000 to 1:2,000 (Amersham Biosciences, Needham, MA, USA). The binding results were visualized by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA). The primary antibodies are summarized in Table I.

Immunohistochemical staining. Tissues were fixed with 10% buffered formalin, embedded in paraffin and decalcified in 10% EDTA solution. Representative blocks were then cut to 4 μm, deparaffinized with xylene, and rehydrated in a series of ethanol washes (100, 90, 80 and 70%). Sections were then incubated with 3% H_2O_2 and 5% serum to block endogenous peroxidase activity and non-specific binding. For the APOBEC3B protein, sections were incubated with anti-human APOBEC3B antibody. The sections were then incubated with the biotinylated secondary antibodies and visualized by DAB. Counterstaining was carried out with hematoxylin. The sections were dehydrated in alcohol and coverslipped. For the negative controls, PBS replaced the primary antibody.

Table I. The antibodies used in the western blot analysis.

Protein	Manufacturer	Catalog no.	Dilution
AKT	Santa Cruz Biotechnology	sc-5298	1:500
p-AKT		sc-135650	1:500
Caspase 3		sc-65495	1:200
Caspase 8		sc-56070	
Caspase 9		sc-8355	1:200
β-actin		sc-103656	1:1,000

Statistical analysis. All experiments were performed in triplicate, and the results are expressed as the means ± standard deviation (SD). Kaplan-Meier survival plots were generated and comparisons were carried out with log-rank statistics. A P-value <0.05 was considered to indicate a statistically significant result. All the statistical analyses and graphics were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Assessment of levels of APOBEC3B mRNA and protein in 52 human chondrosarcoma specimens. Western blotting and immunohistochemical staining were carried out to investigate the protein levels of APOBEC3B in the chondrosarcoma specimens of grade I, II and III, respectively. As shown in Fig. 1A and C, the level of APOBEC3B protein in the chondrosarcoma tissues was higher than the level in the normal tissues (P<0.05). To examine the relationship between the level of APOBEC3B protein and the level of *APOBEC3B* transcription, real-time PCR of *APOBEC3B* mRNA was carried out in the chondrosarcoma specimens. The results showed that the level of *APOBEC3B* mRNA was also higher in the chondrosarcoma specimens than the level in the normal tissues and coincident with the level of protein (P<0.05, Fig. 1B). The levels of *APOBEC3B* mRNA and protein were higher in the cancer tissues of grade III than levels in tissues of grade I or II (P<0.05, Fig. 1). Kaplan-Meier analysis showed that APOBEC3B expression was correlated with the unfavorable prognosis of patients with grade I, II and III stage chondrosarcoma (P<0.05, Fig. 1D).

Effects of APOBEC3B knockdown on biological phenotypes of chondrosarcoma cells. SW1353, OUMS-27 and RUNX3-positive SW1353 cells were transfected with the APOBEC3B shRNA plasmid, and expression of APOBEC3B was determined by western blotting and immunofluorescence analysis. As shown in Fig. 2, the results of the western blot analysis and immunofluorescence analysis confirmed decreased

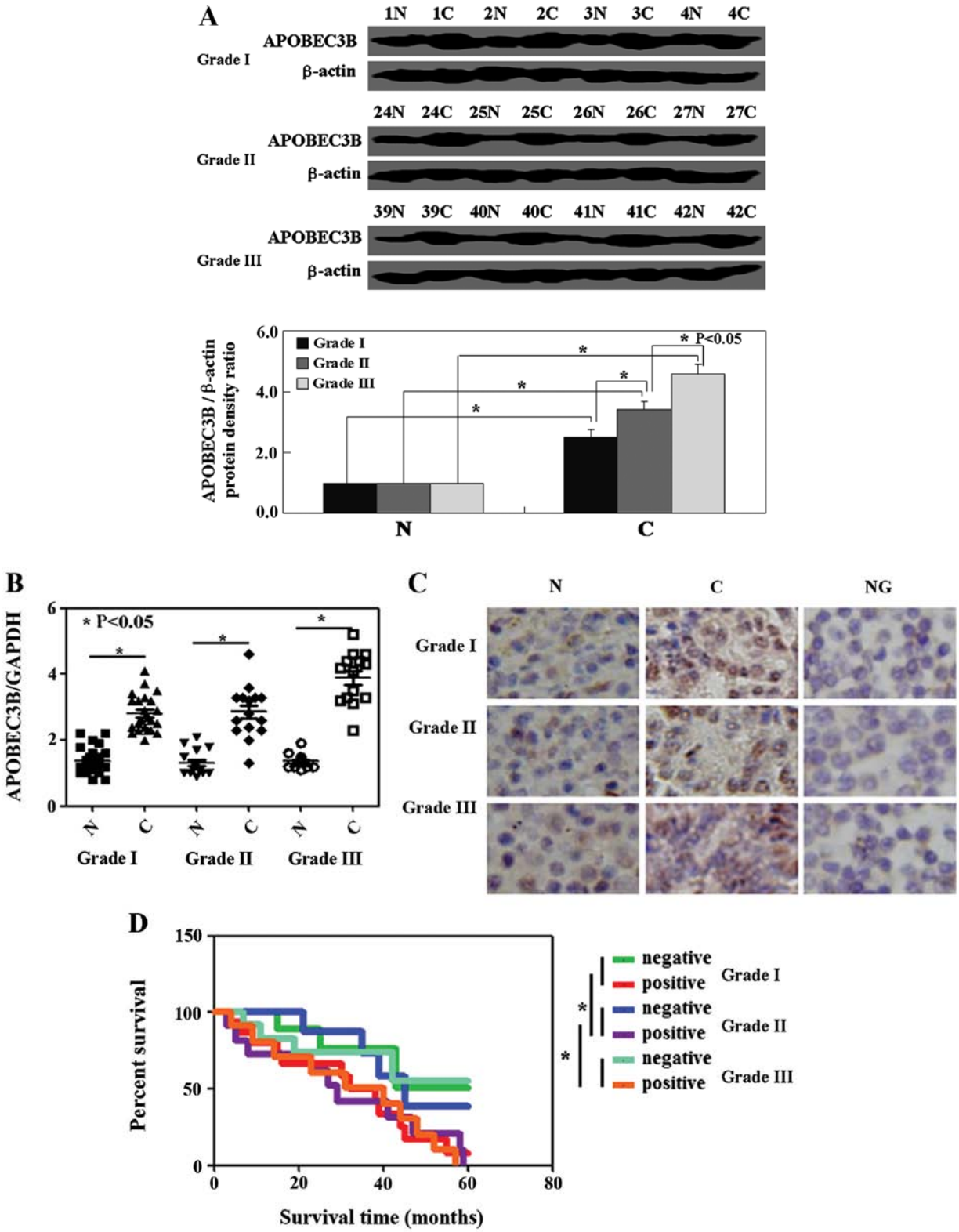

Figure 1. Levels of APOBEC3B protein and mRNA were determined in chondrosarcoma tissue specimens using (A) western blotting and (B) real-time PCR. (C) Immunohistochemical staining for APOBEC3B protein in specimens. The nuclei were counterstained with hematoxylin. (D) Overall survival of the chondrosarcoma patients categorized according to APOBEC3B protein expression and tumor grade as determined by the Kaplan-Meier method. N, normal, C, cancer, NG, negative control.

APOBEC3B protein levels in the three cell lines after transfection.

The results from the colony formation assay showed that the proliferation rates of the SW1353 and OUMS-27 cells after transfection were slightly lower than the rates in the untreated cell lines (P<0.05, Fig. 3A). The proliferation rate of the RUNX3-positive SW1353 cells was lower than the rate in the RUNX3-negative cells (P<0.05, Fig. 3A). Notably,

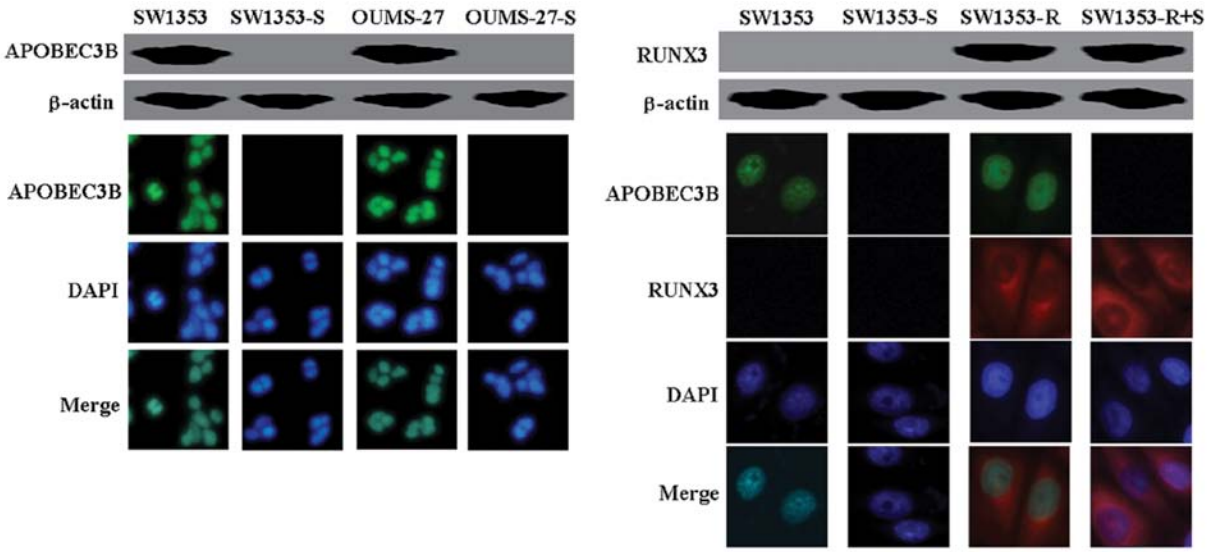

Figure 2. Confirmation of APOBEC3B knockdown in the chondrosarcoma cells. Western blotting and immunofluorescence analysis were used to detect APOBEC3B and RUNX3 proteins in SW1353, OUMS-27 and RUNX3-positive SW1353 cells, respectively. SW1353-S, SW1353 cells transfected with the APOBEC3B shRNA plasmid; OUMS-27-S, OUMS-27 cells transfected with the APOBEC3B shRNA plasmid; SW1353-R, RUNX3-positive SW1353 cells; SW1353-R+S, RUNX3-positive SW1353 cells transfected with the APOBEC3B shRNA plasmid.

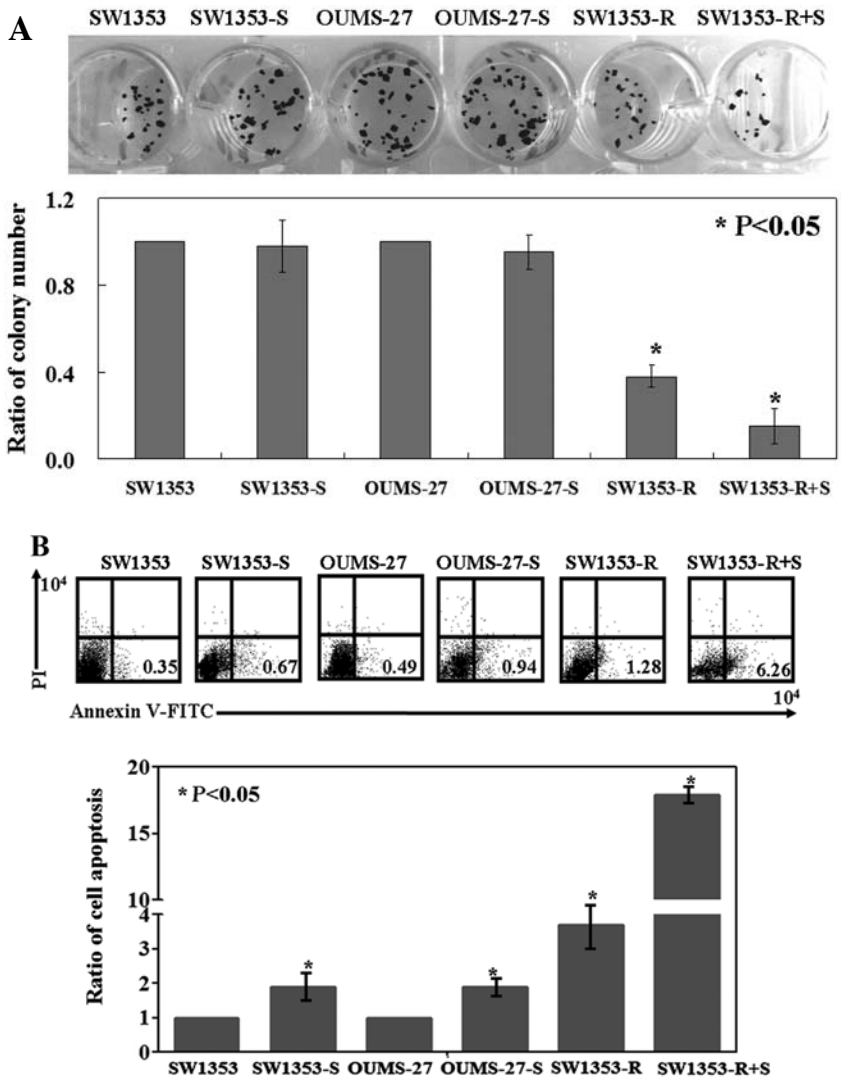

Figure 3. Effects of APOBEC3B knockdown on chondrosarcoma cells. (A) The percentage of growth inhibition following downregulation of APOBEC3B in chondrosarcoma cells was determined by colony formation assay in comparison with the untreated cells. (B) Apoptosis ratio in chondrosarcoma cells was analyzed by double staining with Annexin V/propidium iodide (PI).

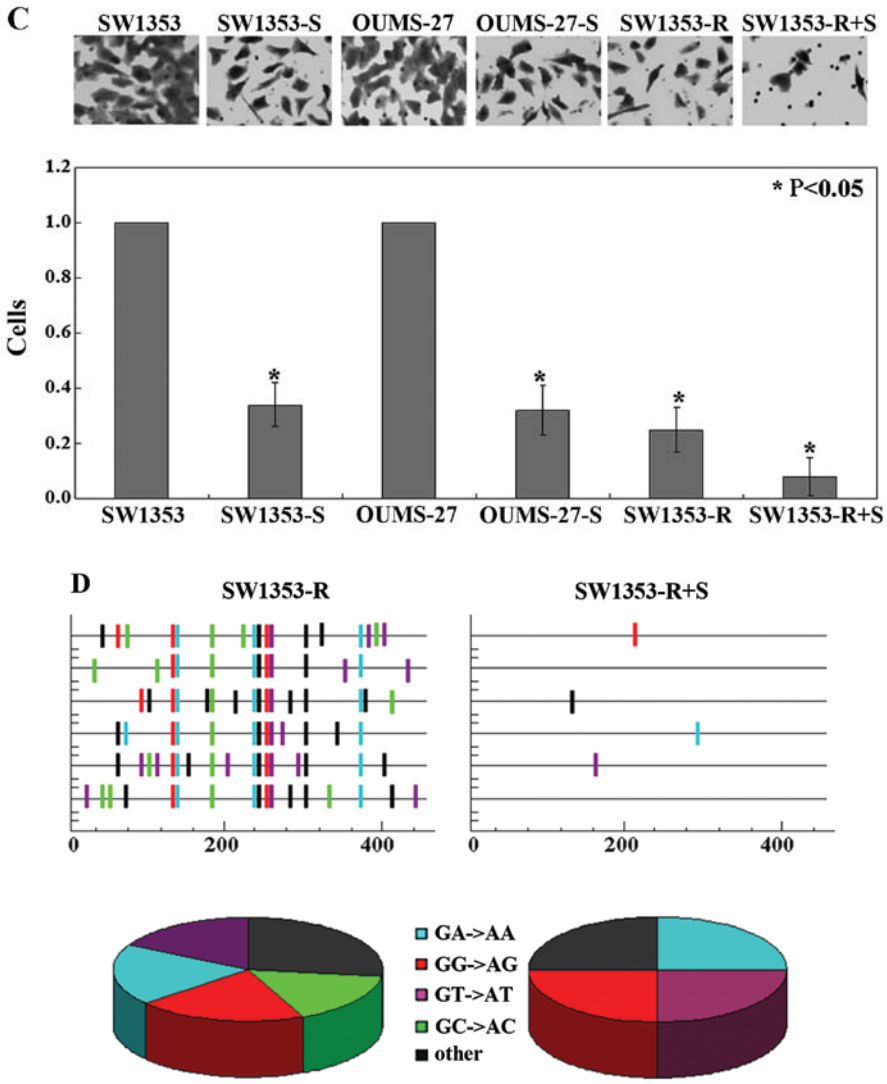

Figure 3. Continued. (C) Migrated cells found on the bottom side of the membrane were fixed and stained. The number of migrated cells on the membrane bottom were counted. (D) APOBEC3B-induced editing in RUNX3. PCR products amplified at the lowest denaturing temperature from chondrosarcoma cells were sequenced. The mutations are indicated by color as follows: GA-to-AA mutations (blue lines), GG-to-AG mutations (red lines), GT-to-AT mutations (pink lines), and GC-to-AC mutations (green lines). SW1353-S, SW1353 cells transfected with the APOBEC3B shRNA plasmid; OUMS-27-S, OUMS-27 cells transfected with the APOBEC3B shRNA plasmid; SW1353-R, RUNX3-positive SW1353 cells; SW1353-R+S, RUNX3-positive SW1353 cells transfected with the APOBEC3B shRNA plasmid.

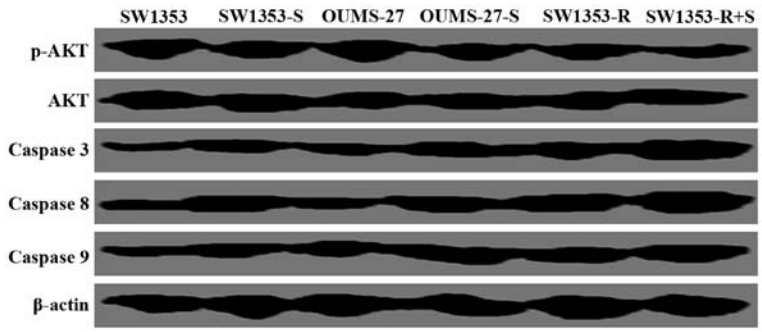

Figure 4. Mechanisms of APOBEC3B and RUNX3 in chondrosarcoma cells. Western blot analysis was performed to detect apoptosis-related proteins. Cell lysates were electrophoresed and AKT, p-AKT active caspase-9, active caspase-8 and active caspase-3 proteins were detected by their specific antibodies. SW1353-S, SW1353 cells transfected with the APOBEC3B shRNA plasmid; OUMS-27-S, OUMS-27 cells transfected with the APOBEC3B shRNA plasmid; SW1353-R, RUNX3-positive SW1353 cells; SW1353-R+S, RUNX3-positive SW1353 cells transfected with the APOBEC3B shRNA plasmid.

the RUNX3-positive SW1353 cells following APOBEC3B shRNA plasmid transfection had the lowest proliferative rate among the cell lines (P<0.05, Fig. 3A). The percentage of apoptotic cells in each group was determined by Annexin V

and PI double-staining. Correspondingly, the RUNX3-positive SW1353 cells after APOBEC3B shRNA plasmid transfection exhibited increased apoptosis (6.26±0.42%) compared to the percentage of apoptosis in the SW1353 (0.35±0.09%) and RUNX3-positive SW1353 cells (1.28±0.16%) ($P<0.05$, Fig. 3B). Furthermore, a significantly decreased mobility of SW1353 and OUMS-27 cells following APOBEC3B shRNA plasmid transfection was noted when compared to the untreated cells ($P<0.05$, Fig. 3C). We next assessed whether the reduced antitumor activities of RUNX3 are correlated with mutagenesis. The RUNX3-positive SW1353 cells with APOBEC3B shRNA did not yield any PCR products amplified at lower denaturing temperatures, suggesting that no editing took place. The RUNX3-positive SW1353 cells exhibited extensive mutagenesis in the presence of APOBEC3B (Fig. 3D).

In the western blot analysis, a decreased level of p-AKT and increased levels of caspase-3, -8 and -9 were detected in the RUNX3-positive SW1353 cells transfected with APOBEC3B shRNA when compared with the levels in the untreated cells (Fig. 4), while total levels of AKT showed no changes (Fig. 4).

Discussion

The *APOBEC3* gene family encodes proteins that play pivotal roles in intracellular defense against viral infection (8). APOBEC3B is overexpressed in many types of tumor tissues and lymphoma cells (15,16). In the present study, we found that APOBEC3B was overexpressed in chondrosarcoma tissues and cell lines. Furthermore, we also confirmed that APOBEC3B expression was correlated with an unfavorable prognosis of the chondrosarcoma patients.

Previous studies found that restoration of RUNX3 induces cell cycle arrest and apoptosis (14,17). Importantly, in the present study, we found that APOBEC3B knockdown induced slight apoptosis in the chondrosarcoma cells. However, the RUNX3-positive SW1353 cells with APOBEC3B knockdown had a higher apoptotic ratio than the cells without APOBEC3B knockdown. The APOBEC3 genes have been shown to deaminate 5-methylcytosine and 5-hydroxymethylcytosine, with base excision repair of the resulting mismatch providing a mechanism for active DNA demethylation (8). There are numerous reports of APOBEC3 deaminase editing-independent restriction of HIV, including APOBEC3-mediated reduction of reverse transcription activity, strand transfer, or integration (18,19). To the best of our knowledge, no related studies have shown the effects of APOBEC3 on mutation of antitumor genes. Mutation signatures have aided in the identification of environmental mutagens and carcinogens (20). C→T transitions in cervix, bladder, lung, head and neck, and breast cancers have been suggested to be caused by APOBEC3B (21). In the present study, we found that the reduced antitumor activity of RNUX3 was caused by APOBEC3B. Furthermore, we found that RUNX3 inhibited p-AKT expression. The clinical and prognostic significance of AKT and its activated form (p-AKT) in human cancer have been investigated (11).

In conclusion, this study provides evidence that APOBEC3B interferes with RUNX3 transcription. This may, at least in part, contribute to RUNX3-mediated inhibition of chondrosarcoma cell invasion and proliferation. We are currently investigating whether other genes in chondrosarcoma are also regulated by APOBEC3B.

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