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Original article

Epigenetic regulation of the pro-apoptosis gene *TSSC3* in human osteosarcoma cells

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ABSTRACT

Promoter hypermethylation can lead to a loss of genetic imprinting in carcinogenesis. The mechanism for the loss of expression of the imprinted gene *TSSC3* has not been investigated in cases of osteosarcoma. In this study, we treated osteosarcoma cell lines with 5-Aza-CdR, which is a widely-used DNA methyltransferase inhibitor, and found dose-dependent reduction in cell growth, conversion of cell morphology to a non-motile phenotype, and obvious increase in apoptosis. In addition, we also found that 5-Aza-CdR reactivated *TSSC3* expression through demethylation of the promoter regions. These findings indicate that the *TSSC3* gene is silenced through hypermethylation of the promoter regions, a mechanism commonly associated with gene silencing in cancer. Finally, we examined the role of *TSSC3* in human osteosarcoma SaOS2 cells. We showed that *TSSC3* overexpression suppressed SaOS2 cell growth and increased apoptosis through caspase-3 upregulation, thereby, suggesting that *TSSC3* may play a pro-apoptosis role to maintain the normal balance of growth. Taken together, these observations suggest that the epigenetic regulation of *TSSC3*, a pro-apoptosis gene, provides valuable insights into possible osteosarcoma therapies.

1. Introduction

Osteosarcoma is the most common type of malignant bone tumor in teens. High potential for invasion and early development of distant metastasis to the lungs are the main causes of mortality in osteosarcoma patients. Although the combination of neoadjuvant chemotherapy plus surgery has been used for the treatment of osteosarcoma in recent years, there has been no obvious increase in the survival rate [1].

Previous studies have identified genetic alterations that play a part in the development and progression of these malignant bone tumors [2,3]. Recently, genomic imprinting, which is an epigenetic form of gene regulation leading to the expression of only one parental allele, has also been shown to contribute to carcinogenesis [4]. We previously examined the expression of imprinted genes in osteosarcoma cells by using oligonucleotide microarrays, and the gene expression profiles indicated that most of the cells exhibited changes in *TSSC3* gene expression [5]. *TSSC3* is also called *PHLDA2*, which is the first apoptosis-related gene found to be involved in imprinting [6]. The loss of *TSSC3* expression has been reported in several cases of malignant tumors [7–11], but the regulation mechanisms responsible for the expression and the function of *TSSC3* in osteosarcoma are not well understood.

DNA methylation at CpG sites is involved in the control of imprinting in humans. Aberrant DNA methylation of cytosine residues disturbs the function of imprinted genes, and may be involved in malignant transformation [12]. This misregulation leads to the activation of oncogenes and the silencing of the expression of the tumor suppressor gene in cancer. Unlike genetic events, DNA methylation appears to be a reversible process, and this reversibility provides a novel approach for cancer therapy [13]. 5-Aza-CdR, which is a widely-used DNA methyltransferase inhibitor, has shown antitumor properties in a variety of tumors, including head and neck carcinoma, colorectal carcinoma, ovarian carcinoma, and malignant melanoma [14–16]. However, thus far, the activity of 5-Aza-CdR in osteosarcoma has not been fully elucidated compared to other tumors.

Therefore, in this study, we explored the effects of 5-Aza-CdR on the growth, migratory phenotype, and apoptosis of the human osteosarcoma cell line SaOS2, analyzed the relationship between *TSSC3* expression and the methylation status of promoter regions, and investigated the potential function of *TSSC3* in osteosarcoma cell line SaOS2. Our results show that 5-Aza-CdR has antitumor properties and reactivates *TSSC3* expression by demethylation. In addition, we found that *TSSC3* overexpression in SaOS2 cells may be responsible for growth suppression and apoptosis induction, and is associated with increased caspase-3 expression.

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2. Materials and methods

2.1. Cell culture

The human osteosarcoma cell lines SaOS2, U2OS, and OS9901 were obtained from the American Type Culture Collection, and were maintained in RPMI-1640 (Gibco) with 10% fetal calf serum (PAA) at 37 °C in a 5% CO_2 incubator. 5-Aza-CdR (Sigma) was dissolved in phosphate buffer solution (PBS) and stored at –80 °C as a 1000× stock solution. Cells were cultured in the absence or the presence of varying concentrations of 5-Aza-CdR for 72 h in order to examine morphology, cell proliferation, and apoptosis assays.

2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay

Cell proliferation was determined by MTT assay, according to a previous study. In brief, 5×10^3 cells/well were added to 96-well plates and cultured for 72 h. Then, 20 μL of MTT solution (5 mg/mL in PBS) was added to 200 μL medium in the plates and incubated at 37 °C for 4 h. After removal of the staining solution, 100 μL dimethyl sulfoxide (DMSO) was added and the absorbance was measured at 490 nm with an ultraviolet spectrophotometer (Bio-RAD, USA).

2.3. Apoptosis assay

An apoptosis assay was performed with an Annexin V-FITC Kit (Jingmei, China) according to the manufacturer's instructions. Briefly, SaOS2 cells were harvested at 80% confluence, and then; 1×10^6 cells/mL were resuspended in 200 μL binding buffer that contained 10 μL Annexin V-FITC (20 μg/mL) and 5 μL propidium iodide (PI; 50 μg/mL). After incubation for 15 min at room temperature, 300 μL of the binding buffer was added. Stained cells were subsequently analyzed by flow cytometry.

2.4. Actin staining assay

SaOS2 cells were cultured on coverslips for 72 h, fixed with 4% paraformaldehyde, and then blocked with normal goat serum (Zhongshan, China). After washing, the cells were incubated with 5 mg/L FITC-conjugated phalliodin (Sigma, USA) at 37 °C for 30 min, and the nuclei were counterstained with Hoechst 33258 (Beyotime, China) at room temperature for 15 min. The cells were examined under a confocal laser-scanning microscope (Leica, Germany).

2.5. RNA preparation, RT–PCR and Real-time RT–PCR

Total RNA was isolated from SaOS2 cells using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA by Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA). Subsequent reverse transcription polymerase chain reactions (RT–PCR) were used to analyze *TSSC3* transcripts (NM_001753). The housekeeping gene β-actin was used as an expression control. The sequences of the primers used for RT–PCR included: *TSSC3*: 5′-ACCGCCTGAGCCTGTTCCC-3′ and 5′-CTGGCGGCTGCGAAAGTCC-3′ (annealing at 57 °C, 242 bp); and β-actin: 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′ (annealing at 55 °C, 540 bp).

The amplified RT–PCR products were electrophoresed with ethidium bromide on a 1% agarose gel and analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR for the analysis of caspase-3 (NM_032991) was carried out with a 1:10 dilution of cDNA on an ABI Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA), using the primers: 5′-GCGAATCAATGGACTCTGGAAT-3′ and 5′-AGGTTTGCTGCATCGACATCTG-3′ (annealing at 56 °C, 151 bp). The results were analyzed as previously described [5].

2.6. Western blot analysis

SaOS2 cells were lysed in lysis buffer [10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% Triton-X, and complete protease inhibitor mixture (Roche, Germany)], and centrifuged at 15,000 × *g* for 1 h at 4 °C in order to remove the insoluble materials. Total protein was then measured by a BCA protein assay kit (Pierce Protein Research Products, Rockford, IL, USA). Approximately 30 μg of total protein was separated by 10% SDS–polyacrylamide gels and transferred onto a nitrocellulose membrane (Invitrogen). Membranes were blocked with 5% fat-free milk for 2 h at room temperature, and subsequently incubated overnight at 4 °C with a mouse monoclonal antibody against human *TSSC3* at a 1:300 dilution (Abcam, England) or a rabbit polyclonal antibody against human β-actin at a 1:5000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing with TBS-T, the membranes were incubated at 37 °C for 1 h with an appropriate secondary antibody (anti-mouse or anti-rabbit IgG at a 1:2000 dilution; Zhongshan, China). Immunoreactivity was detected by an ECL Kit (Amersham Pharmarcia Biotech) according to the instructions of the manufacturer.

2.7. Bisulfite sequencing array

Genomic DNA from SaOS2 cells was isolated using a DNA kit (Omega, USA) and modified by EZ DNA methylation kit (Zymo, USA), according to the manufacturer's directions. Bisulfite-treated DNA was used as a template for PCR amplification. The promoter region of *PHLDA2* (8701–10,000 bp of NC000011.8) was analyzed using the primers: 5′-AAGGAAGAAGGGTAAATAAAAT-3′ and 5′-AAACCAACAACCCATAAAACTC-3′ (270 bp). The reactions were conducted by LA Taq polymerase with CG buffer (TaKaRa, China), and performed as follows: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 57 °C for 45 s, 72 °C for 30 s, and a 72 °C extension for 8 min. To analyze the methylation state of each CG site, the PCR products were gel-purified using a gel extraction kit (Omega, USA) and subsequently ligated into the pMD18-T vector (TaKaRa, Japan). Individual plasmid molecules from positive recombinant clones were isolated and sequenced by the ABI PRISM 377 DNA Sequencer at Invitrogen Biotech Company (Shanghai, China).

2.8. TSSC3 construction, sequencing, cell transfection, MTT assays, and apoptosis assays

The sequence information of human *TSSC3* was obtained from Genbank (NM_001753), and the CDS of *TSSC3* was amplified from a pOTB7 vector (Sanying Biotechnologies, China) with the primers: 5′-CGGAAGCTTATGAAATCCCCCGAC-3′ and 5′-GCGGGATCCTCATGGCGTGCGGGGTT-3′ for 35 cycles with 57 °C annealing. After the pEGFP-C3 vector and PCR products were digested with *Hin*dIII and *Bam*H1, the two fragments were ligated. Positive recombinant clones were isolated and sequenced by the ABI PRISM 377 DNA Sequencer at Invitrogen Biotech Company (Shanghai, China). MTT assay and apoptosis assay on SaOS2 cells were carried out by transfection cells with equimolar amounts of pEGFP-C3-TSSC3 and pEGFP-C3 vectors by FuGENE HD transfection reagent (Roche, Germany), according to the manufacturer's instructions.