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Che-1 gene silencing induces osteosarcoma cell apoptosis by inhibiting mutant p53 expression

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ABSTRACT

The transcriptional cofactor Che-1 is an RNA polymerase II (Pol II) which is involved in tumorigenesis, such as breast cancer and multiple myeloma. Che-1 can also regulate mutant p53 expression, which plays roles in many types of cancer. In this study, we aimed to investigate the effects and specific mechanism of Che-1 in the regulation of osteosarcoma (OS) cell growth. We found that Che-1 is highly expressed in several kinds of OS cells compared with osteoblast hFOB1.19 cells. MTT and flow cytometry assays showed that Che-1 depletion by siRNA markedly suppressed MG-63 and U2OS cell proliferation and promoted apoptosis. The chromatin immunoprecipitation (ChIP) assay verified the presence of Che-1 on the p53 promoter in MG-63 and U2OS cells carrying mutant p53. Further studies showed that Che-1 depletion inhibited mutant p53 expression. Notably, our study showed that the loss of Che-1 inhibits proliferation and promotes apoptosis in MG-63 cells by decreasing the level of mutant p53. Therefore, these findings open the possibility that silencing of Che-1 will have therapeutic benefit in OS.

1. Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, and in recent years, patients with OS have been mostly incremental approximately one to three cases annually per million worldwide [1,2]. OS arises primarily in adolescents and is a leading cause of cancer death in adolescents; it also has a second peak of incidence in those over the age of 50 [3]. The prognosis of OS is poor due to its metastatic dissemination to the bone and lung. The five-year survival rate for patients with OS has increased to 60–70% with the combined application of surgical techniques and multi-agent chemotherapy [4,5]. Although some advances in surgical techniques and multi-agent chemotherapy treatment have been achieved, most strategies have limited efficacy in the treatment of OS [6]. Recently, some evidence indicated that OS is accompanied by genomic instability and a high frequency of kataegis and chromothripsis; however, the recurrent targetable mutations and trials of targeted agents for OS has not been very well clarified [1]. Thus, it is urgent to develop additional potential molecular target to, further contribute to the treatment of osteosarcoma [7].

Che-1, also known as apoptosis-antagonizing transcription factor (AATF), was initially identified as an interacting protein for RNA polymerase II (Pol II) subunit 11 [8]. The human Che-1 gene is located at chromosome 17q11.2-q12 and contains 560 amino acids. The protein has a leucine zipper structure which mediates protein–protein interactions [9]. Che-1 has been highly conserved during evolution, and plays pivotal roles in cell cycle progression governed by fundamental networks and gene transcription [8,10,11]. Ample evidence suggests that Che-1 may participate in gene transcriptional regulation and associated with a wide range of cellular pathways, such as cell proliferation and survival under pathological and physiological conditions [12,13]. Accompanied with the pro-proliferative role of this protein, Che-1 also shows obvious anti-apoptotic activity [13]. Recently, as an important component of the DNA damage response (DDR), evidence indicated that Che-1 is part of the DDR machinery which maintains genome integrity and prevents tumorigenesis [13]. Desantis suggested that Che-1 may regulate the activity of p53 and also found that Che-1 is a promising yet attractive drug target for cancer therapy [14]. Another recent study found that Che-1 promotes tumor cell survival by sustaining mutant p53 (mtp53) transcription and inhibiting DDR activation [15].

Abbreviations: OS, osteosarcoma; ChIP, chromatin immunoprecipitation; AATF, apoptosis-antagonizing transcription factor; Pol II, polymerase II; DDR, DNA damage response; FBS, fetal bovine serum.

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The tumor suppressor p53 protein is a sequence-specific transcription factor that regulates DNA repair, senescence, cell-cycle arrest and the expression of apoptosis-associated target genes; it also inhibits angiogenesis and metastasis [16]. p53 is the most frequent mutated gene in various types of human cancers; these mutations are usually missense mutations within the DNA-binding domain, resulting in the up-regulation of full-length mutant p53 protein [17,18]. Mtp53 proteins are abundantly present in various cancer cells [19], and unlike the tumor-suppressor function of wild type p53 gene, mtp53 acquires novel oncogenic properties to promote tumorigenesis [17,20]. Indeed, it is well-accepted that mtp53 is associated with abnormal cell cycling, drug resistance, metastasis and invasion in human cancers [21]. Emerging evidence suggests that mtp53 promotes ovarian cancer cell adhesion to mesothelial cells, and the p53-R273H mutant mediates cancer cell survival and anoikis resistance [17,22]. However, to the best of our knowledge, there is no report of an investigation into the regulatory relationship between Che-1 and p53 mutants in OS cells. Thus, in this study, we investigated whether Che-1 affects the level of a p53 hotspot mutant, p53-R273H, which plays a role in the survival and apoptosis of osteosarcoma cells.

2. Experimental procedures

2.1. Cell culture and transfection

Human osteosarcoma (OS) cell lines MG-63, SoSP-9607, Saos-2, U2OS and osteoblast hFOB1.19 cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (Gibco; Invitrogen; Life Technologies, Germany) containing 10% fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml). pCMV p53-R273H and the control pCMV expression vector were obtained from Addgene (Cambridge, MA, USA). All cells were maintained in 5% CO_2 at 37 °C and were transfected using Lipofectamine™ 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.2. Cell viability assay

Cell survival was measured by the MTT assay. Briefly, cells were transfected as described above, and seeded into 24-well plates at 3×10^5 cells/well, then incubated for different time periods (24, 48, 72 and 96 h). Cell viability assay was carried out using an MTT kit (Roche, Switzerland) according to the manufacturer's procedures.

2.3. Apoptosis assay

Apoptosis was measured by flow cytometry (Biovision, Milpitas, CA, USA) with Annexin V-FITC and PI staining. Cells were transfected with pCMV p53-R273H, harvested and incubated with Annexin V-FITC and PI for 15 min to obtain double staining. After incubation in the dark for 30 min, cells were subjected to flow cytometry for apoptosis analysis.

2.4. RT-PCR

Che-1 and P53 mRNA levels in OS cells were detected based on the work of other laboratories with minor modification [23]. Total RNA was isolated from OS cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with the Thermo Script RT-PCR kit (Invitrogen). GAPDH was used as an internal control. The following human specific primers were employed in PCR amplifications [15]: Che1 forward 5′-CCGGAATTCGGATAAGACCAAA- CTGGCT-3′; Che1 reverse 5′-CCGCTCGAGGAGTTCTCGAAGGAGCTG-3′; p53 forward 5′-ATGGAGGAGCCGCAGTCAGA-3′p53, reverse 5′-AGAAGCCCAGA- CGGAAACCG-3′; GAPDH forward 5′- CCAAGGTCATCCATGACAAC-3′, reverse 5′-TTACTCCTTGGAGGCCATGT-3′.

2.5. Western blot

Cells were lysed and equal amounts of proteins (40 μg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used in this study: mouse monoclonal primary antibodies against anti-p53 (DO1, Sigma), β-actin (Sigma), anti-p-Che1-474 (PhosphoSolution, Aurora, CO, USA), and a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). An enhanced chemiluminescence reagent was used to visualize protein expression; GAPDH was used as the internal control.

2.6. RNA silencing

Che-1 silenced cells were constructed according to the method of Desantis [24], with minor modifications. The 22-nucleotide siRNA for Che-1-1 (nucleotides 1062–1083) and Che-1-2 (nucleotides 1473–1492), and siRNA for the negative control GFP (nucleotides 122–143) sequence were synthesized by using the Silencer siRNA construction kit (Ambion) following the manufacturer's procedure.

2.7. Chromatin immunoprecipitation assay (ChIP)

ChIP assays in MG-63 and U2OS cells were performed according to a previously described assay with minor modifications [15,24]. An anti-Che-1 antibody was used, and IgG with no specific immunoglobulins was used as the negative control. Generally, for quantitative CHIP analysis, purified DNA was amplified using promoter-specific primers: p53 promoter forward 5′-GGGAGAAAACGTTAGGGTGTGG-3′ and p53 promoter reverse 5′-AAGCGTGTCACCGTCGTGGAAA-3′.

2.8. Statistical analysis

All data in the present study are shown as the mean ± standard deviation (SD) of three individual experiments carried out in triplicate. Statistical analysis was performed by one-way ANOVA followed with Student's t-test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Che-1 expression is markedly up-regulated in OS cells

At first, we aimed to evaluate the expression of Che-1 in various OS cell lines (MG-63, SoSP-9607, Saos-2, and U2OS) and hFOB1.19 osteoblasts. RT-PCR and western blot were used to measure the mRNA and protein expression of Che-1 in these cells, respectively. As shown in Fig. 1A and B, the mRNA and protein expression of Che-1 was dramatically increased in MG-63, SoSP-9607, Saos-2 and U2OS cells with respect to the levels observed in normal hFOB1.19 cells. To further assess the contributory role of Che-1 in OS cells, Che-1 siRNA was used to silence the expression of Che-1 in MG-63 and U2OS cells. The results showed that siRNA against Che-1-1 and Che-1-2 both markedly reduced the level of Che-1 in MG-63 and U2OS cells (Fig. 1C and D, $P < 0.05$).