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ASPL-TFE3 Oncoprotein Regulates Cell Cycle Progression and Induces Cellular Senescence by Up-Regulating p21

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Abstract

Alveolar soft part sarcoma is an extremely rare soft tissue sarcoma with poor prognosis. It is characterized by the unbalanced recurrent chromosomal translocation der(17)t(X;17)(p11;q25), resulting in the generation of an ASPL-TFE3 fusion gene. ASPL-TFE3 oncoprotein functions as an aberrant transcriptional factor and is considered to play a crucial role in the tumorigenesis of alveolar soft part sarcoma. However, the underlying molecular mechanisms are poorly understood. In this study, we identified p21 ($p21^{WAF1/CIP1}$) as a direct transcriptional target of ASPL-TFE3. Ectopic ASPL-TFE3 expression in 293 cells resulted in cell cycle arrest and significant increases in protein and mRNA levels of p21. ASPL-TFE3 activated p21 expression in a p53-independent manner through direct transcriptional interactions with the p21 promoter region. When ASPL-TFE3 was expressed in human bone marrow–derived mesenchymal stem cells in a tetracycline-inducible manner, we observed the up-regulation of p21 expression and the induction of senescence-associated β-galactosidase activity. Suppression of p21 significantly decreased the induction of ASPL-TFE3-mediated cellular senescence. Furthermore, ASPL-TFE3 expression in mesenchymal stem cells resulted in a significant up-regulation of proinflammatory cytokines associated with senescence-associated secretory phenotype (SASP). These results show that ASPL-TFE3 regulates cell cycle progression and induces cellular senescence by up-regulating p21 expression. In addition, our data suggest a potential mechanism by which ASPL-TFE3-induced senescence may play a role in tumorigenesis by inducing SASP, which could promote the protumorigenic microenvironment.

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Introduction

Alveolar soft part sarcoma (ASPS) is an extremely rare sarcoma with unclear origins, comprising 0.5% to 1.0% of all soft tissue sarcomas [1]. Although ASPS typically presents as a slow-growing tumor, its overall survival rate is poor because of chemoresistance and the high incidence of pulmonary and brain metastases during the early stages of this disease [2–4]. Moreover, almost all cases of ASPS have the unbalanced recurrent chromosomal translocation der(17)t(X;17)(p11;q25), which results in generation of the ASPL-TFE3 (also known as ASPSCR1-TFE3) fusion gene [5]. Because the ASPL gene is joined in frame with either the third (type 1) or fourth (type 2) exon of TFE3, two types of fusion transcripts have been identified [5].

TFE3 belongs to the microphthalmia transcription factor-transcription factor E (MiTF-TFE) basic helix–loop-helix leucine zipper transcription factor family, which binds to the E-box DNA consensus sequence CANNTG [6,7]. In a subset of renal cell carcinomas with the translocation Xp11.2, TFE3 fuses with PRCC, CLTC, PSF, NonO, PARP14, LUC7L3, KHSRP, and DVL2, resulting in the expression of PRCC-TFE3, CLTC-TFE3, PSF-TFE3, NonO-TFE3, PARP14-TFE3, LUC7L3-TFE3, KHSRP-TFE3, and DVL2-TFE3 fusion oncogenes, respectively [8–14]. In addition, ASPL-TFE3-positive

Abbreviations: ASPS, alveolar soft part sarcoma; ChIP, chromatin immunoprecipitation; FACS, fluorescence-activated cell sorting; MSCs, mesenchymal stem cells; OIS, oncogene-induced senescence; SA-β-gal, senescence-associated β-galactosidase; SASP, senescence-associated secretory phenotype

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renal cell tumors have also been reported [15]. The cellular roles of ASPL have only been partially characterized, but the mouse homolog TUG is a tethering protein that forces the retention of GLUT4-containing vesicles in the cytoplasm in the absence of insulin [16].

The ASPL-TFE3 fusion gene retains the DNA binding and activation domain of TFE3, whereas the N-terminal region of TFE3 is replaced with ASPL sequences [5]. The ASPL-TFE3 oncoprotein is believed to play crucial roles in the progression of ASPS. ASPL-TFE3 functions as an aberrant transcriptional factor and induces the inappropriate up-regulation of various molecules that contribute to the pathogenesis and progression of ASPS [17]. Indeed, several TFE3 fusion oncoproteins, including ASPL-TFE3, up-regulate the Met receptor tyrosine kinase gene and induce oncogenic phenotypes such as uncontrolled cell proliferation, invasion, and metastasis [18]. However, the molecular roles of ASPL-TFE3 are poorly understood.

Deregulated cell proliferation is critical to tumor formation and progression. Because TFE3 plays roles in the regulation of cell growth [19–21], we hypothesized that ASPL-TFE3 may affect the proliferation of tumor cells by inducing inappropriate expression of cell cycle regulatory proteins. Therefore, we investigated the effects of ASPL-TFE3 on the cell cycle machinery. In this paper, we report that ASPL-TFE3 affected the cell cycle machinery by the direct transcriptional up-regulation of p21. In addition, we reveal that the expression of ASPL-TFE3 in mesenchymal stem cells (MSCs) induced p21-mediated cellular senescence and increased the mRNA level of proinflammatory cytokines.

Materials and Methods

Cells

HeLa human cervical carcinoma cells, 293 human embryonic kidney cells, KATO III human gastric cancer cells, and UE7T-13 human bone marrow-derived MSCs, which were immortalized using retroviruses expressing human papillomavirus protein E7 and human telomerase catalytic subunit (hTERT) [22], were obtained from Health Science Research Resource Bank (Osaka, Japan). Cells were maintained under 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum.

Tissue Sample

Frozen tissue was obtained from a patient who underwent surgical treatment for ASPS. Written informed consent was obtained for tissue to be used in research. This study was approved by the Ethics Committee of Tottori University.

Cloning and Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from frozen tissue of ASPS patient using ISOGEN (Nippon GENE, Tokyo, Japan) according to the manufacturer's instructions and a cDNA clone encoding human ASPL-TFE3 was subsequently amplified by reverse transcription polymerase chain reaction (RT-PCR) using a Takara PrimeScript High Fidelity RT-PCR kit (Takara, Otsu, Japan) with the following primers: forward, 5′-AAGCTTCACCATGGCGGCCCCGG CAGGC-3′ (including HindIII site and Kozak sequence), and reverse, 5′-TCACTTGTCGTCATCGTCTTTGTAGTCGGA CTCCTCTTCCATGCT-3′ (including FLAG epitope tag sequence and STOP codon). Amplified fragments were inserted into the pGeMT-easy vector (Promega, Madison, WI) and DNA was sequenced using ABI PRISM (Applied Biosystems, Foster City, CA). DNA sequencing confirmed the identity of the type I fusion gene of ASPL-TFE3. Subsequently, ASPL-TFE3 cDNA was digested using HindIII and NotI and inserted into the tetracycline-inducible expression vector pcDNA4/TO (Invitrogen, Carlsbad, CA) to produce pcDNA/TO-AT.

Transfection and Selection of Clones

For the tetracycline-regulated system (T-Rex system; Invitrogen), 293 and UE7T-13 cells were transfected with pcDNA6/TR (Invitrogen) and either empty pcDNA4/TO vector or pcDNA/TO-AT using FuGENE HD transfection reagent (Roche, Mannheim, Germany), according to the manufacturer's instructions, and these were designated 293/TR-Vec, 293/TR-AT, UE7T/TR-Vec, and UE7T/TR-AT cells, respectively. After 24 h, cells were placed in media containing 5 μg/ml blasticidin (Invitrogen) and 125 μg/ml Zeocin (Invitrogen), and drug-resistant colonies were isolated. Tetracycline (1 μg/ml; Invitrogen) was added to the growth media to induce of ASPL-TFE3 (tested by RT-PCR and western blotting).

Cell Growth Assay

293/TR-AT and UE7T/TR-AT cells were seeded at densities of 1×10^5 and 2×10^4 cells per well, respectively, in 6 well plates. Tetracycline or phosphate buffered saline (PBS) was added to the media and cells were cultured for designated times. Subsequently, cells were trypsinized and counted using a trypan blue dye exclusion test.

Fluorescence-Activated Cell Sorting Analysis

Cells were trypsinized, harvested, and fixed at −20°C in 70% ethanol for at least overnight. Fixed cells were stained with 1 ml of propidium iodide (PI) solution (0.05% NP-40, 50 μg/ml propidium iodide, and 10 μg/ml RNase A) for at least 2 h at 4°C. Stained cells were analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter, Brea, CA).

Western Blot

Cells were suspended in lysis buffer (0.1% SDS, 40 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml leupeptin), and were then centrifuged. Lysate samples containing 10 to 20 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). PVDF membranes were then incubated overnight at 4°C with primary antibodies and signals were detected using Pierce Western Blotting Substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Antibodies against p21, p27, p16, and cyclin dependent kinase (Cdk) 2 were obtained from BD Transduction Laboratories (Franklin Lakes, NJ). Antibodies against FLAG and actin were purchased from Sigma (St Louis, MO). Rb antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cdk4 antibody was obtained from Epitomics (Burlingame, CA). Phosphorylated-Rb (Ser807/811) antibody was purchased from Cell Signaling Technology (Beverly, MA).

Plasmids

The p21 promoter (−2264 to +11 base pairs) was obtained from the DNA Bank, RIKEN BioResource Center (Ibaraki, Japan) and was subcloned into the pGV-B2 vector (Toyo Ink, Tokyo, Japan).

Luciferase Reporter Assay

Cells were plated on 35-mm culture dishes at a density of 2×10^5 per dish. On the next day, the cells were transfected with 0.5 μg of the