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Research paper

The deubiquitinating enzyme activity of USP22 is necessary for regulating HeLa cell growth

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ABSTRACT

Ubiquitin-specific protease 22 (USP22) can regulate the cell cycle and apoptosis in many cancer cell types, while it is still unclear whether the deubiquitinating enzyme activity of USP22 is necessary for these processes. As little is known about the impact of USP22 on the growth of HeLa cell, we observed whether USP22 can effectively regulate HeLa cell growth as well as the necessity of deubiquitinating enzyme activity for these processes in HeLa cell. In this study, we demonstrate that USP22 can regulate cell cycle but not apoptosis in HeLa cell. The deubiquitinating enzyme activity of USP22 is necessary for this process as confirmed by an activity-deleted mutant (C185S) and an activity-decreased mutant (Y513C). In addition, the deubiquitinating enzyme activity of USP22 is related to the levels of BMI-1, c-Myc, cyclin D2 and p53. Our findings indicate that the deubiquitinating enzyme activity of USP22 is necessary for regulating HeLa cell growth, and it promotes cell proliferation via the c-Myc/cyclin D2, BMI-1 and p53 pathways in HeLa cell.

1. Introduction

Ubiquitin-specific protease (USP), a subfamily of deubiquitinating enzymes (DUBs) (D'Andrea and Pellman, 1998; Reyes-Turcu and Wilkinson, 2009), is responsible for the removal of ubiquitin or polyubiquitin from target proteins, the processing of ubiquitin precursors, and the disassembly of unanchored polyubiquitin by catalyzing the hydrolysis of isopeptide bonds in ubiquitin-protein conjugates (Takayama and Toda, 2010). The growth of many cancers is regulated by USPs: USP8 is related to non-small cell lung cancer (Baykara et al., 2013); USP28 is a potential prognostic marker for bladder cancer (Guo et al., 2014); and USP9X expression correlates with tumor progression and poor prognosis in esophageal squamous cell carcinoma (Peng et al., 2013). Therefore, USPs are important factors for cancer. Recent observations have identified an 11-gene poly-comb/cancer stem cell signature that could predict the likelihood of treatment failure in cancer patients (Glinsky, 2005). Ubiquitin-specific protease 22 (USP22) is a new putative cancer stem cell marker involved in the 11-gene polycomb/cancer stem cell signature (Zhang et al., 2008a,b). It belongs to a large family of proteins with ubiquitin hydrolase activity. USP22 is a key subunit of the human Spt-Ada-Gcn5 acetyltransferase (hSAGA) transcriptional cofactor complex. Within hSAGA, USP22 regulates the transcription of downstream genes related to epigenetic alteration and cancer progression by removing ubiquitin from histone H2A and H2B (Zhang et al., 2008b).

Moreover, USP22 can regulate tumor recurrence, distant metastasis, therapeutic failure and poor prognosis via its functions in some cell signaling pathways: USP22 regulates the cell cycle via the c-Myc/cyclin D2 pathway and down-regulation of p15 and p21 expression in HepG2 cells (Ling et al., 2012); USP22 silencing was also found to lead to reduced expression of cell cycle proteins, including CDK1, CDK2 and Cyclin B1 in human brain glioma cells (Li et al., 2013); USP22 may act as an oncogene in human colorectal cancer as it positively regulates the cell cycle via both the BMI-1-mediated INK4a/ARF pathway and the Akt signaling pathway (Liu et al., 2012); USP22 antagonizes p53 transcriptional activation by deubiquitinating sirt1 to suppress cell apoptosis (Lin et al., 2012). The siRNA-mediated knock-down of USP22 could effectively induce cell cycle arrest by regulating target molecules, such as cyclin D2, p21, p15 and p53, and could also inhibit cell growth. Elevated expression of USP22 can predict shorter intervals of tumor recurrence, distant metastasis, therapeutic failure and poor prognosis in patients with many types of cancer such as colorectal cancer (Liu et al., 2010, 2011), breast cancer (Y. Zhang et al., 2011), gastric cancer (Yang et al., 2011) and others (Ueda et al., 2015).

USP22 can regulate the growth and prognosis of many cancers, but little is known about the impact of USP22 on the growth of human cervical cancer cell lines, and whether the deubiquitinating enzyme activity of USP22 is necessary for these effects is not clear. In this

Abbreviations: USP, ubiquitin-specific protease; DUBs, deubiquitinating enzymes; USP22, ubiquitin-specific protease 22; qRT-PCR, quantitative real-time polymerase chain reaction.

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study, we used siRNA to specifically suppress expression of USP22, and we observed that the knock-down of USP22 could effectively inhibit HeLa cell proliferation and induce cell cycle arrest. We also suggest that USP22 may regulate cell cycle progression by down-regulating p53 expression and up-regulating cyclin D2, BMI-1, c-MYC expression. The deubiquitinating enzyme activity of USP22 is necessary for regulating HeLa cell growth.

2. Materials and methods

2.1. Cell culture antibodies and reagents

The human HeLa cell line was purchased from American Type Culture Collection (ATCC). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FBS, GIBCO) and 1% penicillin–streptomycin at 37 °C in a 5% CO_2 atmosphere. The USP22 antibody (1:500) was from Abcam (UK). β-actin (1:5000) was from Proteintech (USA). Lipofectamine 2000 was from Invitrogen Corp (USA). The reverse-transcription polymerase chain reaction (RT-PCR) kit and primers were from Takara (Japan).

2.2. Transfection of siRNA

HeLa cells were seeded in 6-well plates at a concentration of 4×10^5 cells/2 ml in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. After 24 h, the medium was replaced with fresh medium without antibiotics. Meanwhile, the cells were transfected with siRNA or a negative control oligonucleotide using Lipofectamine RNAiMAX according to the manufacturer's instructions. After incubation for 4–6 h, the medium containing the Lipofectamine RNAiMAX complexes was replaced with fresh DMEM containing 10% FBS. Then, the cells were cultured for subsequent experiments. All siRNAs were obtained from Invitrogen Corp (USA), and the three specific sequences for silencing were: human USP22 siRNA-1, sense 5′-GCU GUU UCA CAA AGA AGC AUA UUC A-3′, and anti-sense 5′-UGA AUA UGC UUC UUU GUG AAA CAG C-3′; siRNA-2, sense 5′-CAG CAG CCC ACG GAC AGU CUC AAC A -3′, and anti-sense 5′-UGU UGA GAC UGU CCG UGG GCU GCU G 3′; siRNA-3, sense 5′-GCC AAG UCC UGU AUC UGC CAU GUC U-3′, and anti-sense 5′-AGA CAU GGC AGA UAC AGG ACU UGG C-3′. The transfection efficiency was assessed by transfection of negative control siRNA. The negative control siRNA sequence was: 5′-UUC UCC GAA CGU GUC ACG UTT ACG UGA CAC GUU CGG AGA ATT-3′. The effect of RNA interference was analyzed by qRT-PCR and western blot analyses.

2.3. Molecular cloning of USP22

The pcDNA3.1-USP22-V5-FLAG plasmid was a gift from Boyko S. Atanassov. The pcDNA3.1-USP22-V5-FLAG plasmid was digested by *NotI* and *XhoI*, and then USP22 was inserted via blunt-end ligation. The expression vector pGEX-6p-1 (Amersham Pharmacia Biotech) was digested by *SalI* and filled in to generate blunt ends. Then, the pGEX-6p-1-USP22 plasmid was produced by inserting the complete coding sequence of USP22 into the pGEX-6p-1 plasmid. The pGEX-USP22 plasmid was digested by *NotI* and *BamHI*, and then USP22 was inserted via blunt-end ligation. The expression vector pAC-T7 plasmid was digested by *BamHI* and filled in to generate blunt ends. Then, a pAC-T7- USP22 plasmid was produced by inserting the complete coding sequence of USP22 into the pAC-T7 plasmid.

2.4. Deubiquitinating enzyme activity assay of hUSP22

The USP cleavage assays used ubiquitin-met-β-galactosidase (Ub-Met-β-gal) and pGEX-Ub52 (GST-Ub52) (W. Zhang et al., 2011) as model substrates. Total protein extracts of the Ub-Met-β-gal group were analyzed by western blotting, and total protein extracts of the pGEX-Ub52 group were purified by glutathione agarose beads and detected by Coomassie Brilliant Blue. The pGEX-6p-1-USP46 and pAC-T7-USP46 plasmids expressing USP46 were used as positive controls for the USP cleavage assay. The relative intensity of the resulting bands was analyzed by the Odyssey V3.0 software.

2.5. Site-directed mutagenesis and deubiquitinating enzyme activity assay

Cys 185, which is located within the conserved Cys-box of USP22, was mutated to serine. Arg 98 was mutated to tryptophan, Asn 283 was mutated to serine, Pro 290 was mutated to leucine, and Tyr 513 was mutated to cysteine. The resulting plasmids were pGEX-USP22 (C185S), pGEX-USP22 (R98W), pGEX-USP22 (N283S), pGEX-USP22 (P290L) and pGEX-USP22 (Y513C), respectively. Mutations were confirmed by DNA sequencing. The deubiquitinating enzyme activity assay method was consistent with the method used for USP22 wild type (WT).

2.5.1. Flow cytometry analysis

HeLa cells were seeded in 6-well plates at a concentration of 4×10^5 cells/2 ml in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. After 24 h, the medium was replaced with fresh medium without antibiotics. Meanwhile, the cells were transfected with siRNA, negative control siRNA, pEGFP-C1, pEGFP-USP22 (WT), pEGFP-USP22 (C185S) and pEGFP-USP22 (Y513C) using Lipofectamine RNAiMAX and Lipofectamine 2000 according to the manufacturer's instructions. After incubation for 48 h, the cells were collected for flow cytometric analysis. For the apoptosis and cell-cycle analyses, $1–2 \times 10^6$ cells were stained with propidium iodide and analyzed with a Becton–Dickinson FACSort analyzer and Cell Quest software (BD Biosciences).

2.5.2. Transfection of USP22 and SNPs, RNA extraction and qRT-PCR analysis

HeLa cells were seeded in 6-well plates at a concentration of 4×10^5 cells/2 ml in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. After 24 h, the medium was replaced with fresh medium without antibiotics. Meanwhile, the cells were transfected with siRNA, negative control siRNA, pEGFP-C1, pEGFP-USP22 (WT), pEGFP-USP22 (C185S) and pEGFP-USP22 (Y513C). After incubation for 48 h, total RNA was extracted from the cells using Trizol (Invitrogen, USA) and reverse transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The Trans Start Top Green qPCR Super Mix (TransGen Biotech, Beijing, China) was used for real-time quantitative PCR. The mRNA levels of BMI-1, p53, c-Myc and cyclin D2, as well as that of the internal standard β-actin were measured by qRT-PCR in triplicate using the Rotor Gene 6000 real-time detection system (Bio-Rad).

2.6. Statistical analysis

All statistical analyses were performed by using the SPSS software version 17.0. Statistical analysis was performed using Student's t-test and Wilcoxon rank sum test. Differences were considered statistically significant when p values were <0.05.

3. Results

3.1. Suppression of USP22 Expression in HeLa cells by siRNA transfection

Three siRNAs (siRNA-1, siRNA-2 and siRNA-3) were used to silence the expression of USP22 in HeLa cells. The effect of the transfection was determined by qRT-PCR and western blot analysis (Fig. 1). The results of our study showed that the USP22 siRNA-1 was the most efficient in silencing the expression of USP22 (Fig. 1A, B), and it was optimally